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OLIGONUCLEOTIDE THERAPIES FOR RNA AND DNA
Modulation of natural splice-variants, DNA structure & characterization of new synthetic nucleotides and reporter cell lines

Cristina S. J. Rocha

Karolinska Institutet

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Oligonucleotide Therapies for RNA and DNA – Modulation of natural splice-variants, DNA structure & characterization of new synthetic nucleotides and reporter cell lines

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Cristina S. J. Rocha

Principal Supervisor:
Associate Professor Rula Zain
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Research Centre

Co-supervisors:
Professor C. I. Edvard Smith
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Research Centre

Dr. Karin E. Lundin
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Research Centre

Opponent:
Professor Jørgen Kjems
University of Aarhus
Department of Molecular Biology and Genetics

Examination Board:
Professor Camilla Sjögren
Karolinska Institutet
Department of Cell and Molecular Biology

Associate Professor Anna-Lena Ström
Stockholms University
Department of Neurochemistry

Professor Mats Eriksson
Karolinska Institutet
Department of Medicine, Huddinge

To my grandmother Cristina

The women whose life teach me never to give up, to always fight and suppress the pain, to always be honest and humble.

"For a successful technology, reality must take precedent over public relations, for Nature cannot be fooled"

Richard P. Feynman

"What do you care what other people think?

Further adventures of a curious character" (New York, 1988)

ABSTRACT

Oligonucleotide therapy is an evolving field having shown fast and important developments in the last years. From genetics to metabolic, inflammatory, immunodeficiency diseases, cancer and viral infections the medical applications for this type of therapy are becoming broader every day. However, the major challenge for these therapies is still the delivery; thus new chemistries, as well as improved delivery vectors are needed. Equally, the lack of relevant reporter systems hampers the characterization and progress of these emergent "drugs."

This thesis work was aimed to address some of the gaps presented above. Thus, the introduction section starts with a brief overview of the gene therapy. It continues with an explanation of the oligonucleotide therapy strategies and technologies used for RNA and DNA targeting and ends with a clinical case for each approach. The methodology section explains the theoretical and practical aspects of the most relevant techniques in this study and the results section explain the rational and gives a brief presentation of the main results and conclusions for each paper.

Paper I presents a new splice-switching approach for treating diseases by regulating proteins at the splicing level. The new principle was to convert the normal splice form to a natural, but less abundant and inactive, splice variant to treat hypercholesterolemia. Paper II shows the development and characterization of a new collection of reporter cell lines for splice-switching. These reporter cell lines can serve as models for cell-type-specific screenings of different ON chemistries and delivery vectors. Paper III and IV demonstrate the development and characterization of a new nucleic acid chemical modification providing an oligomer with cell penetrating properties. Finally, in paper V a new mechanism for gene expression regulation at the genome level is presented.

LIST OF SCIENTIFIC PAPERS

RNA therapeutics inactivate PCSK9 by inducing a unique intracellular retention form.
Cristina S. J. Rocha, Oscar P. B. Wiklander, Lilian Larsson, Pedro M. D. Moreno, Paolo Parini, Karin E. Lundin, C.I. Evard Smith
Journal of Molecular and Cellular Cardiology. 2015: 82, 186-93

Four novel splice-switch reporter cell lines: distinct impact of oligonucleotide chemistry and delivery vector on biological activity.
Cristina S. J. Rocha, Karin E. Lundin, Mark A. Behlke, Rula Zain, Samir EL Andaloussi, C.I. Edvard Smith
Nucleic Acid Therapeutics. September 2016, ahead of print.

Nuclease resistant oligonucleotides with cell penetrating properties.
Stefan Milton, Dmytro Honcharenko, **Cristina S. J. Rocha**, Pedro M. D. Moreno, C. I. Edvard Smith and Roger Strömberg
Chemical Communications. 2015: 51(9), 4044-4047

Fully and partially AECM-modified oligonucleotides. Synthesis and initial studies on cellular uptake and splice-switching activity in different reporter cell lines.
Dmytro Honcharenko[#], **Cristina S. J. Rocha[#]**, Jyotirmoy Maity, Ulf Tedebark, Stefan Milton, Rula Zain, C. I. Edvard Smith and Roger Strömberg
Manuscript

Disruption of higher order DNA structures in Friedreich's ataxia (GAA)n repeats by PNA and LNA targeting.
Helen Bergquist, **Cristina S. J. Rocha**, Rubén Álvarez-Asencio, Chi-Hung Ngyuen, Mark. W. Rutland, C. I. Edvard Smith, Liam Good, Peter E. Nielsen, Rula Zain
PLoS One. 2016 (re-submitted, after review)

[#] These authors contributed equally to this work.

Other publications by the author not included in this thesis:

Development of bis-locked nucleic acid (bisLNA) oligonucleotides for efficient invasion of supercoiled duplex DNA.
Pedro M. D. Moreno, Sylvain Geny, Y. Vladimir Pabon, Helen Bergquist, Eman M. Zaghloul, **Cristina S. J. Rocha**, Iulian I. Oprea, Burcu Bestas, Samir EL Andaloussi, Per T. Jørgensen, Erik B. Pedersen, Karin E. Lundin, Rula Zain, Jesper Wengel and C. I. Edvard Smith.
Nucleic Acids Research. 2013: 41(5), 3257-3273.

CONTENTS

LIST OF ABBREVIATIONS

§	Section
2′-MOE	2′-*O*-methoxyethyl
2’-OMe	2’-*O*-methyl
3’ ss	3’ splice site
3D	Three-dimensional
5’ ss	5’ splice site
AFM	Atomic force microscopy
Ago2	Argonaute-2
ATP	Adenosine triphosphate
BQQ	Benzoquinoquinoxaline
BQQ-OP	Benzoquinoquinoxaline-1,10-phenanthroline
CLCN1	Chloride channel protein 1
CPP	Cell penetrating peptide
CRISPR	Clustered regularly interspaced short palindromic repeats
DM1	Myotonic dystrophy disease
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified Eagle medium
DMPK	Dystrophia myotonica protein kinase
ds	Double-stranded
DSI	Double-strand invasion
EGS	External guide sequence
EM	Electron microscopy
EM	Electronic microscopy
ESE	Exonic splicing enhancer
ESE	Exonic splicing enhancers
ESS	Exonic splicing silencers
FBS	Fetal bovine serum
FH	Familial hypercholesterolemia
FRDA	Friedreich’s ataxia
FXN	*Frataxin*

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HCMV	Human cytomegalovirus
HPRT	Hypoxanthine phosphoribosyltransferase 1
ISE	Intronic splicing enhancers
ISS	Intronic splicing silencers
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LN	Lipid nanoparticle
LNA	Locked nucleic acid
miRNA	micro RNA
mRNA	Messenger RNA
MS	Multiple sclerosis
NLS	Nuclear localisation signal
ON	Oligonucleotide
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
PMO	Phosphorodiamidate morpholino oligomer
PNA	Peptide nucleic acid
PO	Phosphodiester
pre-mRNA	Precursor mRNA
PS	Phosphorothioate
RA	Rheumatoid arthritis
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNase H1	Ribonuclease H1
RNase P	Ribonuclease P
RT-PCR	Reverse transcription polymerase chain reaction
siRNA	Small interfering RNA
SMA	Spinal muscular atrophy
ss	Single-stranded
SSO	Splice-switching oligonucleotide

TALENs	Transcription activator-like effector nucleases
TAR	Trans-activation response
TFO	Triple helix-forming oligonucleotides
TNRC6	Trinucleotide repeat containing 6
TTR	Transthyretin
UNA	Unlocked nucleic acid
UTR	Untranslated regions
ZFNs	Zinc finger nucleases

1 INTRODUCTION

1.1 GENE THERAPY – AN OVERVIEW OVER TIME

In 1971, at least 1500 human diseases were accepted to be genetically driven [1]. For the majority, the molecular basis was unknown and, despite the progress in the biochemical characterization of human genetic diseases, only ninety-two were found to be associated with a genetically induced specific enzyme deficiency [2]. By that time, several scientific advances allowed for better understanding of the structure and function of DNA [3–5], synthesis of nucleotides [6–8], cellular uptake of proteins and DNA by endocytosis [1], and the ability to manipulate DNA *in vitro*.

Gene therapy emerged then, as the idea "that correct exogenous DNA could be used to compensate the defective DNA in the patients with the genetic defect" [1,9,10]. Initially, it would involve approaches like the introduction of DNA either directly or by somatic cell fusion, transfer of genetic material by virus-like particles, infection with active or inactive virus containing genes, or infection with a viral nucleic acid [9]. However, concerns about unknown outcomes were strong, as proven in later years [1,11].

The following years came with significant developments [11] as infectivity improvement of adenovirus [12], the mammalian cells transfection of plasmids with calcium phosphate by Wigler *et al.* [13], and the first western blotting protocol [14]. But also a better understanding of RNA processing [15], the *in vitro* synthesis of the beta globin gene by Maniatis *et al.* [16], and the first hypothesis of messenger RNA (mRNA) splicing by Catterall *et al.* [17].

In 1980, Cline and his colleagues made the first human study, re-infusing bone marrow cells transfected *in vitro* with plasmids containing the human globin gene in thalassaemia patients. The study gave rise to controversy and, in response, the Recombinant DNA Advisory Committee of the NIH created the Gene Therapy Subcommittee to regulate the application of molecular genetic tools for human use [11]. By 1981, the life cycle and transduction capabilities of the retrovirus were unravelled. Immediately, reports appeared describing techniques to increase the genetic information transferred by a retrovirus and the production of vectors with infection efficiency of 100% in human and mammalian cells [11]. The virus became "the vectors". Quickly, numerous studies demonstrating that several cell types could be stably genetically corrected with retroviral vectors increased its applicability in several genetic diseases [11]. In 1989, the first human genetic engineering experiment was approved [18] and in 1990 Blaese *et al.* initiate a gene therapy protocol aiming to correct adenosine deaminase deficiency [11,19–21]. Nowadays, cancer is the most common disease treated by virus-mediated gene therapy, followed by monogenetic, cardiovascular and infectious diseases. In clinical trials, the retroviral and the adenoviral vectors are the gene transfer vectors most common, with adenovirus showing to be a very successful carrier for e.g. haemophilia and retinal diseases. [21].

Parallel to the virus-mediated gene therapies, the oligonucleotide (ON) therapies were also developing, but in a more discreet way, following and/or promoting molecular biology advances [22]. Differently from the virus approach, ONs are designed specifically to modulate gene expression through different mechanisms. All share the same principle: binding of a complementary ON sequence, by Watson-Crick base pair hybridization, to the specific target [23].

The first antisense approach was described in 1978, by Stephenson *et al.* and Zamecnik *et al.*, and consisted of translation inhibition of the Rous sarcoma viral RNA by a sequence complementary oligodeoxyribonucleotide [24,25]. It was followed in 1979 by the discovery of the ribonuclease H1 (RNase H1) specific cleavage of RNA/DNA duplexes [26]. This two reports proved that ON therapeutics could be applied not only as steric-blockers of RNAs (**§ 1.2.3**) but also as inducers of its degradation by an enzymatic process (**§ 1.2.2**). From this point, we observed a boost in ON synthesis of new chemical analogues (**Table 1**, **§ 1.4**), as well as protocols for solid-support [27] and automation synthesis of DNA- [28] or RNA- [29] ONs.

Table 1 - Chronology of chemical nucleotide analogues related to this study.

Year	Chemical Modification	Localization	References
1964	2´-Fluoro	Sugar	[6,7]
1966	Phosphorothioate	Phosphate backbone	[8]
1969	2´-*O*-methyl (2´-OMe)	Sugar	[30]
1989	Phosphorodiamidate Morpholino Oligomer (PMO)	Sugar and phosphate backbone	[31]
1991	Peptide Nucleic Acid (PNA)	Sugar and phosphate backbone replaced	[32]
1998	Locked Nucleic Acid (LNA)	Sugar	[33,34]
1999	2´-*O*-(Carbamoylmethyl)	Sugar	[35]
2002	Tricyclo-DNA	Sugar and phosphate backbone	[36]
2003	2´-*O*-[2-(Amino)-2-oxoethyl]	Sugar	[37]
2015	2´-*O*-(N-(aminoethyl)carbamoyl)methyl (AECM)	Sugar	[38] and Paper IV

Equally, due to the need to improve ON cellular uptake, we witnessed the development of new non-viral vectors and techniques like liposomes, cationic lipids, covalent lipophilic attachments, poly(L-lysine) formulations, asialoglycoprotein conjugates, among others [39,40] (**§ 1.5**).

The possibilities for ONs application were widening, and new mechanisms of action were proven. Respectively, through direct interaction with enzymes, receptors or transcription factors; by inhibition of RNA processing, including RNA structures and splicing [41,42]; inhibition of replication and transcription by binding to duplex DNA [32,43]; and translation arrest by binding to mRNA [39,44] (**§ 1.2**).

In 1993, the first clinical trial Phase I was approved using a phosphorothioate ON complementary to p53 mRNA in patients with acute myelogenous leukaemia or myelodysplastic syndrome [22,45]. In the same year, Dominski and Kole reported the recovery of correct splicing in thalassemic precursor mRNA (pre-mRNA) with a 2′-*O*-methyl (2′-OMe) RNA antisense ONs [41], establishing the splice correction/switching approach (**§ 1.2.3.1**). Equally, the mutagenesis in human cells mediated with triple helix-forming ONs (TFOs) was also reported [46,47] (**§ 1.3.1**). The following advances were the discovery of the RNA interference (RNAi) pathway in 1998 [48,49]; the identification of small interfering RNAs (siRNA) [50]; and the discovery that siRNAs can effectively reduce gene expression in many mammalian cell types [51–55] (**§ 1.2.1**). Soon, the ON technology characterised by a simple concept, rational design, relatively inexpensive cost, and supported by the first sequencing of the human genome in 2001 [56], became essential not only as tools to study gene function but also as therapeutic agents.

From 2010, several protocols based on DNA double-strand break-enhanced homologous recombination [57] emerged [21,58,59]. In these methods, a site-specific cleavage of genomic DNA is required and can be catalysed by zinc finger nucleases (ZFNs) [60–62] or transcription activator-like effector nucleases (TALENs) [63–65]. Recently (2012), the gene editing was revolutionised by a novel technique, the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas)9 system [59,66–68].

Currently, the ON therapeutic field accounts for more than 130 clinical trials listed on ClinicalTrials.gov, three approved antisense drugs (Vitravene™, Macugen™ and Kynamro™) [69], and several others under consideration for market approval in the United States and Europe [70].

1.2 OLIGONUCLEOTIDE THERAPIES – RNA THERAPEUTICS

Oligonucleotide (ON) therapies are broad, involving diverse mechanisms of action and type of targets (RNA and DNA). The focus of this sub-section includes RNA therapeutics and splice-modulating therapies.

RNA therapeutics consist of small synthetic ONs, that target precursor messenger RNA (pre-mRNA) and mRNA. Almost all coding and non-coding RNA sequences are prone to be modulated by synthetic nucleic acids [71]. Three main approaches, involving the binding of complementary ONs to target RNA through base pairing are known (**Figure 1**): RNA interference (RNAi) [48,72,73] (**§ 1.2.1**), antisense ONs [74] (**§1.2.2**) and steric-blocking ONs [75] (**§ 1.2.3**). While RNAi and antisense ONs induce the mRNA enzymatic degradation, steric-blocking ONs act also at the pre-mRNA level and hinder the access of the cellular machinery to RNA without degradation [71,76].

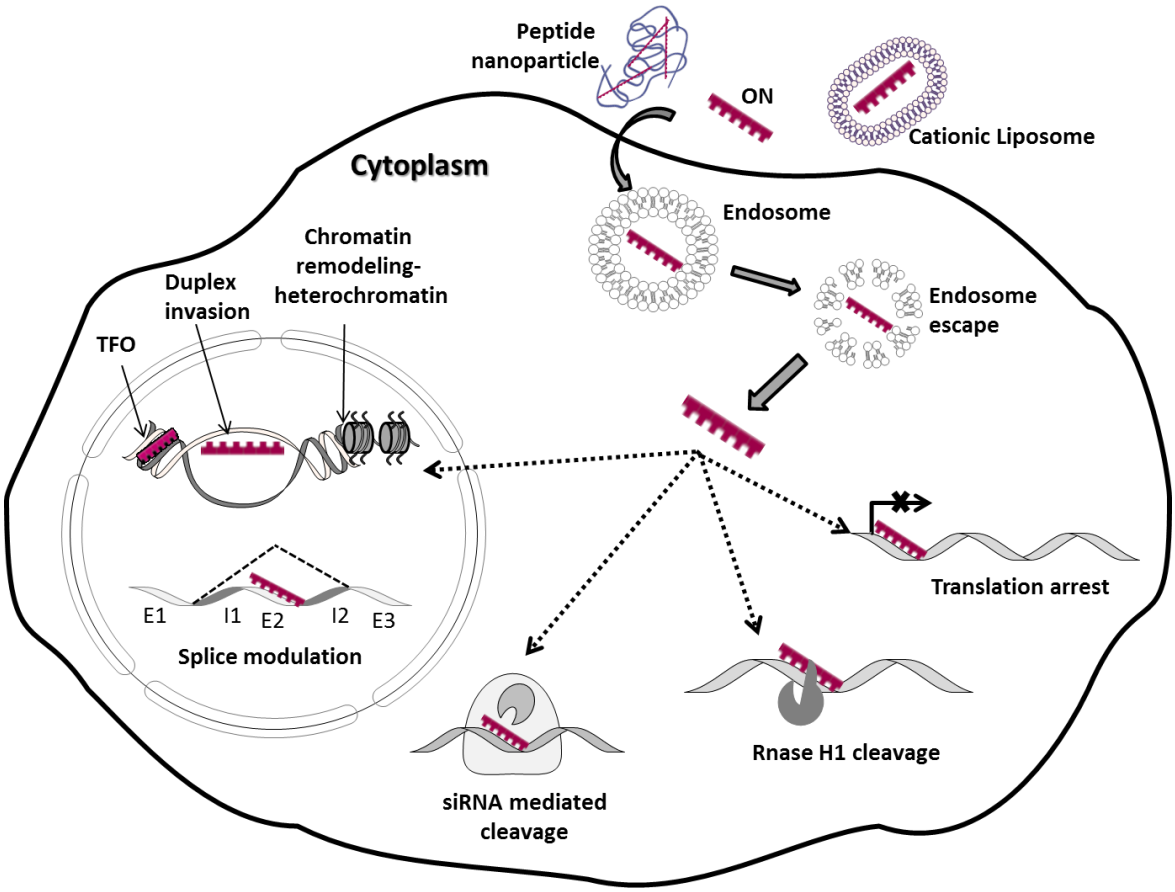

Figure 1: **Mechanisms of action of ON Therapeutics.** ON cellular uptake can be either "naked" or mediated by cationic liposomes or peptide nanoparticles. It can occur via endocytosis, pinocytosis or phagocytosis pathways, which results in an entrapment in the endosome. After release, normally by destabilisation of the endosomal membrane the ON

escapes to the cytoplasm where it can mediate mRNA degradation by siRNA-mediated cleavage or RNase H1 cleavage, block the translation, or move to the nucleus. Here, ON can either bind to the pre-mRNA inducing splice modulation; or bind to the genome to mediate transcription regulation or chromatin remodelling (binding as TFOs, strand invasion of the DNA duplex, and interaction with DNA secondary structures or heterochromatin).

1.2.1 RNA interference

RNAi is a natural cellular process, responsible for regulating gene expression and providing an innate defence mechanism against invading viruses and transposable elements [72]. It was discovered in *Caenorhabditis elegans*, after the delivery of an exogenous, double-stranded RNA (dsRNA) that induced the silencing of the homologous host mRNA [48].

The mechanism that mediates gene silencing involves **1.** Degradation of double-stranded RNA (dsRNA) by endoribonuclease Dicer into small interfering RNAs (siRNAs), consisting of dsRNA fragments of 21–22 nucleotides long. **2.** Interaction of siRNAs with a multiprotein RNA-induced silencing complex (RISC). **3.** Inside the RISC, the two siRNA strands detach. The sense strand is discarded, and the antisense or guide strand binds to mRNA. **4.** When siRNA is fully complementary to its target, the endonuclease Argonaute-2 (Ago2, a component of the RISC) cleaves the mRNA 10 and 11 nucleotides downstream of the 5′ end of the antisense strand [77]. The RNAi pathway is active in the cytoplasm and the nucleus [78]. The stable complex between Ago2 and the trinucleotide repeat containing 6 (TNRC6) family paralogs is conserved in the nucleus and cytoplasm, but accessory proteins differ among them, as showed by Kalantari *et al.* [79], based on orthogonal analysis of mass spectra data.

As RNA therapeutics, synthetic siRNAs (or vectors encoding them) can be designed to target virtually any gene of interest (**Figure 1**). Synthetic siRNAs are double-stranded 21-23 nucleotides ONs that can be introduced into cells, initiating the RNAi pathway without the need of Dicer [51]. However, since RNAi technologies make use of the cell's natural machinery, the compatible interaction between synthetic siRNAs and RISC limits the position and type of chemical modifications responsible for improving nuclease resistance and cell uptake [80]. Consequently, in the antisense strand, phosphorothioate (PS) linkages at the 3′-end and 2′-*O*-methyl (2′-OMe) or 2′-fluoro (2′-F) modifications (**Table 1**, **§ 1.4.1**) in one or two internal nucleotides, are tolerated and improve the resistance of the siRNA to nucleases. The sense strand can be modified to a larger extent without reducing efficacy [77]. Although this approach is promising, several challenges were identified and are under investigation [81], including lack of stability against extracellular and intracellular degradation by nucleases, poor uptake [82], chemical or sequence-related toxicity [83] and off-target effects [75,84].

RNAi machinery also interacts with endogenously encoded short RNA molecules known as microRNAs (miRNAs) [85]. miRNAs are single-stranded RNA (ssRNA) molecules with 21-

25 nucleotides obtained by the Dicer processing of imperfect RNA hairpins [86]. Several genomic regions encode miRNAs, like introns or exons of protein-coding and non-coding regions [87]; repeats regions, like transposons [88]; and pseudogenes [89]. The miRNAs are incorporated into an RISC-like complex and, depending on their degree of complementarity to the target mRNA, promote translational repression or mRNA cleavage. Gene silencing mediated by miRNAs is essential for plant and animal development [90,91], and might also have a role in the pathogenicity of DNA viruses with large genomes that encode miRNAs [92].

While post-transcriptional gene silencing induced by the siRNAs and miRNAs is based on RNA–RNA sequence recognition and base pairing, RNA-DNA interactions are also possible. Consequently, RNAi processes were found to be associated with gene regulation at the genomic level [78,93–101].

siRNAs [84] and miRNAs [87] have been used especially as cancer therapeutics, having currently 17 clinical trials registered for siRNAs and 155 for miRNAs (source https://clinicaltrials.gov/). In parallel, other human genetic, as well as infectious diseases have also been explored, such as Alzheimer, Parkinson, Myopathies, Familial Hypercholesterolemia (FH), Transthyretin (TTR)-mediated amyloidosis [102], Hepatitis C virus infection and Haemorrhagic fevers (especially against Ebola).

1.2.2 Antisense ONs

Antisense ONs, first described as short fragments of unmodified DNA [25], have been subject to several improvements with the incorporation of numerous chemical modifications (**Table 1**, **§ 1.4**). Two antisense drugs are on the market (Vitravene™ for the treatment of cytomegalovirus retinitis and Kynamro™ for the treatment of Homozygous FH), and successful clinical trials are underway [75].

The antisense mechanism is based on the ribonuclease H1 (RNase H1) endonuclease activity to specifically recognise and cleave the RNA strand of the RNA/DNA hybrid substrates [76,103] (**Figure 1**). Based on the structure of human RNase H:RNA/DNA, the protein interacts with eleven base pairs of the hybrid. The enzyme-substrate interactions occur at the minor groove of the RNA/DNA hybrid, and both backbones are moved into respective enzyme grooves. The active site is located in the RNA-binding groove, which interacts with the 2′-OH groups of four consecutive ribonucleotides, two on each side of the phosphate to be cleaved. The two DNA-binding grooves interact with seven DNA nucleotides and, most likely, recognise the DNA strand by the absence of 2′-OH groups and strand conformation, which must be compatible with the distortion induced in the phosphate-binding pocket [104]. The RNA cleavage occurs within or immediately adjacent to the DNA complementary region [26]. Recent studies by Lima *et al.* (2016) show that, after RNase H1 cleavage, the mRNA products are degraded by the cytoplasmic 5′ to 3′ exoribonuclease XRN1, a member of the

RNA surveillance machinery and involved in the deadenylation-independent mechanism associated with nonsense-mediated decay of RNAs [105].

In a similar way to siRNAs (**§ 1.2.1**), not all chemical modifications can be employed in antisense ON design due to the specific structural requirements for RNase H1 activity [106]. Typically, an antisense ON is about 14-22 nucleotides long and has a phosphodiester (PO) or PS backbone [74]. The 2-5 nucleotides at each flank are further modified, normally with 2′-OMe, 2′-*O*-methoxyethyl (2′-MOE), locked nucleic acid (LNA) [33,34] (**Figure 6**, **§ 1.4.2**) or unlocked nucleic acid (UNA) [107] modifications. This design, designated as 'gapmers', is characterised by a 10-nucleotide PS or PO (depending on the flanking modifications) central gap that allows the cleavage of the targeted mRNA by RNase H1 [26,69,75,108–111]. The modified flanks improve the binding between antisense ON and mRNA by increasing the duplex T*m*, allowing better efficiency by reducing the side effects associated with the presence of PS residues, and protects from exonucleases increasing the stability *in vivo* [111].

Unfortunately, antisense ON design is based on a "trial and error" approach due to the inability to predict target accessibility, which is dependent on protein binding or internal RNA secondary structure. However, mRNA regions containing the tetranucleotide GGGA motif are considered as preferred targets for this approach, as reported by Tu *et al.* [112]. Like siRNAs, these ONs present poor intracellular uptake and chemistry-dependent toxicities.

Therapeutically, antisense ONs were tested in more than 100 clinical trials directed to gastrointestinal malignancies; genitourinary and gynecologic cancers; brain, lung and skin malignancies; and hematologic malignancies [84]. Additionally, therapies were also developed for Homozygous FH (Apolipoprotein B) [113,114], Duchenne Muscular Dystrophy (DMD), Multiple Sclerosis (MS), Myasthenia gravis, Hepatitis C, Diabetes, Macular degeneration, surgery related fibrosis and dermal scarring, Rheumatoid arthritis (RA), Crohn's disease, Psoriasis, and Asthma (for more details, review [69,115])

1.2.3 Steric-blocking ONs

Steric-blocking ONs comprehend distinct mechanisms to block RNA and differ from the previous (**§ 1.2.1** and **§ 1.2.2**) by the absence of RNA degradation (**Figure 1**). Depending on the targeted sequence regions at the pre-mRNA or mRNA, the end-products obtained will be different [23,44,75]. The basic requirements for the design of this type of ONs are the resistance to endo- and exonucleases together with specificity and affinity to the target, therefore any chemical modification (**§ 1.4**) that fulfils this requirement can be used.

Accordingly, ONs (designated translation-suppressing ONs) can efficiently inhibit mRNA translation either by disruption of the ribosomes; or by binding regions of mRNA (5' untranslated (5' UTR) [116], 3' untranslated (3' UTR) [117] or translation initiation codon); or affecting elongation steps of protein translation. The control of translation is one of the most important mechanisms for post-transcriptional regulation of gene expression since

determines the final levels of the protein [118]. The initiation of translation [119] is a rate-limiting step of the protein synthesis, controlled by translation -silencing or -enhancing cis-acting elements located in the 5' UTR and 3' UTR of mRNAs [120]. Thus is not surprising the choice of this region as putative targets for ONs. Steric blockade of translation was initially demonstrated by binding of peptide nucleic acid (PNA) oligomers (**Figure 6**, **§ 1.4.3**) to the translation initiation codon region of the Ha-Ras mRNA, with concomitant formation of a truncated product [121,122]. One amazing example of this type of mechanism relates to studies of phosphorodiamidate morpholino oligomer (PMO)-based ONs to decrease Ebola virus in infected monkeys by inhibition of viral mRNA translation (in clinical trials) [123].

On the other side, external guide sequence (EGS), consist of short RNA ONs that bind to mRNA and form a structure recognised by the ribonuclease P (RNase P), which cleaves the mRNA preventing the translation of the protein [75]. This approach is being developed especially for antibacterial and antiviral purposes [124], since RNAi mechanisms (**§ 1.2.1**) cannot be used in prokaryotes. As antibacterial agents, EGS ONs were developed as 11-mer PMOs ON (for expression reduction of the acyl carrier protein in *Escherichia coli*) [125] or as cell penetrating peptides conjugated (CPP) PMOs (PPMOs) (**§ 1.5.2**) (several genes in *E. coli* and *Bacillus subtilis*, with infection reduction) [124]. As antiviral agents, EGS ON was reported to decrease the human cytomegalovirus (HCMV) infection in cells by binding to the mRNA that encodes the protease essential for viral capsid formation and replication [126].

Similarly, ONs can also bind to mRNA sequences involved in secondary structure formation, compromising the proper folding and interfering with mRNA stability or function [23,44]. These structures function as recognition motifs for some proteins, nucleic acids, and ribonucleoproteins that participate in the intermediary RNA metabolism or activities of RNA species. Thus, ONs can prevent 5'-cap formation, dictate the site for polyadenylation and folding of target mRNA [115]. One example is the binding of PS ONs to the trans-activation response (TAR) elements (stem-loop present in all HIV RNA species), disrupting the loop formation and inhibiting the TAR-mediated production of a reporter gene [127,128].

Additionally, ONs can also bind to toxic RNAs avoiding the accumulation of protein factors at expanded triplet repeats [75]. One specific example is the case of myotonic dystrophy disease (DM1), characterised by an expansion of the CTG repeats located in the 3′ UTR of the gene encoding the dystrophia myotonica protein kinase (DMPK). This expansion generates a defective, expanded CUG repeat-containing mRNA, which cannot be correctly spliced and accumulates in the nucleus [129]. It happens that this expanded CUG repeat binds and sequester the splicing factor, muscleblind-like protein 1, which is then unavailable for the splicing of pre-mRNA from several other genes, including the muscle-specific chloride channel protein 1 (CLCN1). The absence of this channel results in the hyperexcitability of the muscle, which causes the myotonia [130]. The interaction between the expanded repeats and the splicing factor can be reversed *in vivo* using a 25-mer (CAG) PMO ON as shown by Wheeler et *al.*[131]. Additional two therapeutic approaches, involving other steric blocking mechanisms, were also developed in DM1. Mulders *et al.* reported, *in vitro* and *in vivo*,

expression silencing of the mutant DMPK RNA and reduction of ribonuclear aggregates using a 21-mer (CAG) 2'-OMe/PS fully modified ON (**Figure 6**, **§ 1.4.1**) in a selective and (CUG)n-length-dependent way [132]. Nakamori *et al.* reported, also *in vitro* and *in vivo*, with two 18-mer fully PS/LNA ONs (**Figure 6**, **§ 1.4.2**), the reduction of CUG expanded transcripts as a consequence of repeat stabilisation at the genome level due to the reduction of R-loops (**Figure 5**, **§ 1.3.3**) formed at the repeat sequence [133].

1.2.3.1 Splice modulating therapies

Splice modulation is also a steric blocking ON mechanism and is mediated by splice-switching or splice correction ONs (SSOs and SCOs, respectively) (**Figure 1**).

Splicing of pre-mRNA is a complex and tightly regulated process, consisting in the concerted removal of the pre-mRNA introns and ligation of exons. This process is catalysed by the spliceosome, a ribonucleoprotein complex [134] highly conserved in eukaryotes, and occurs in almost all of our genes. Splicing of each intron involves two sequential transesterification reactions. The first reaction releases the 5′ exon from the downstream intronic sequence and the formation of a lariat structure. The second reaction releases the lariat from the downstream, 3′ exon and ligates together the 5′ and 3′ exons [135]. In principle, a 5′ splice site can splice to any 3′ splice site, which can result in alternative splicing events [136,137].

Alternative splicing is the mean by which two or more different mRNAs can be produced from a single gene by the alternative inclusion/exclusion of particular sequences of the pre-mRNA. Thus, a single gene can produce protein isoforms with different and even opposite functions [138]. Alternative splicing has been estimated to occur in 40-60% of human genes [139,140]. This high abundance of an occurrence likely explains a certain amount of the functional differences between cell types and suggests that cells can tolerate different isoforms of mRNA and proteins. [141].

Canonical and alternative pre-mRNA splicing requires precision and accuracy to maintain the proper open reading frame, in a way to obtain effective protein production. This high fidelity is dictated by genomic sequences, including 5′ and 3′ splice sites (5′ss and 3′ss); intron branch site; and splice site enhancers and silencers found within both exons and introns. The auxiliary cis-elements, known as exonic or intronic splicing enhancers (ESEs and ISEs) and exonic or intronic splicing silencers (ESSs and ISSs) help in the exon recognition [134,135].

Four classes of pre-mRNA splicing defects are described as causing disease. The cis-acting mutations that disrupt the use of constitutive splice sites; cis-acting mutations that disrupt the use of alternative splice sites; trans-acting mutations that disrupt the basal splicing machinery; and trans-acting mutations that disrupt splicing regulation [138]. The majority of the splice-associated diseases occur through cis-acting mutations.

In a way to modulate pre-mRNA splicing, SSOs or SCOs must block the RNA sequences essential for splicing and prevent the interaction of splicing factors, such as RNA-binding proteins, small nuclear RNAs and other components of the spliceosome, with the pre-mRNA. The mechanisms proposed for the action of ON have been widely revised [75,138,141–143] and can be found schematized in **Figure 2**.

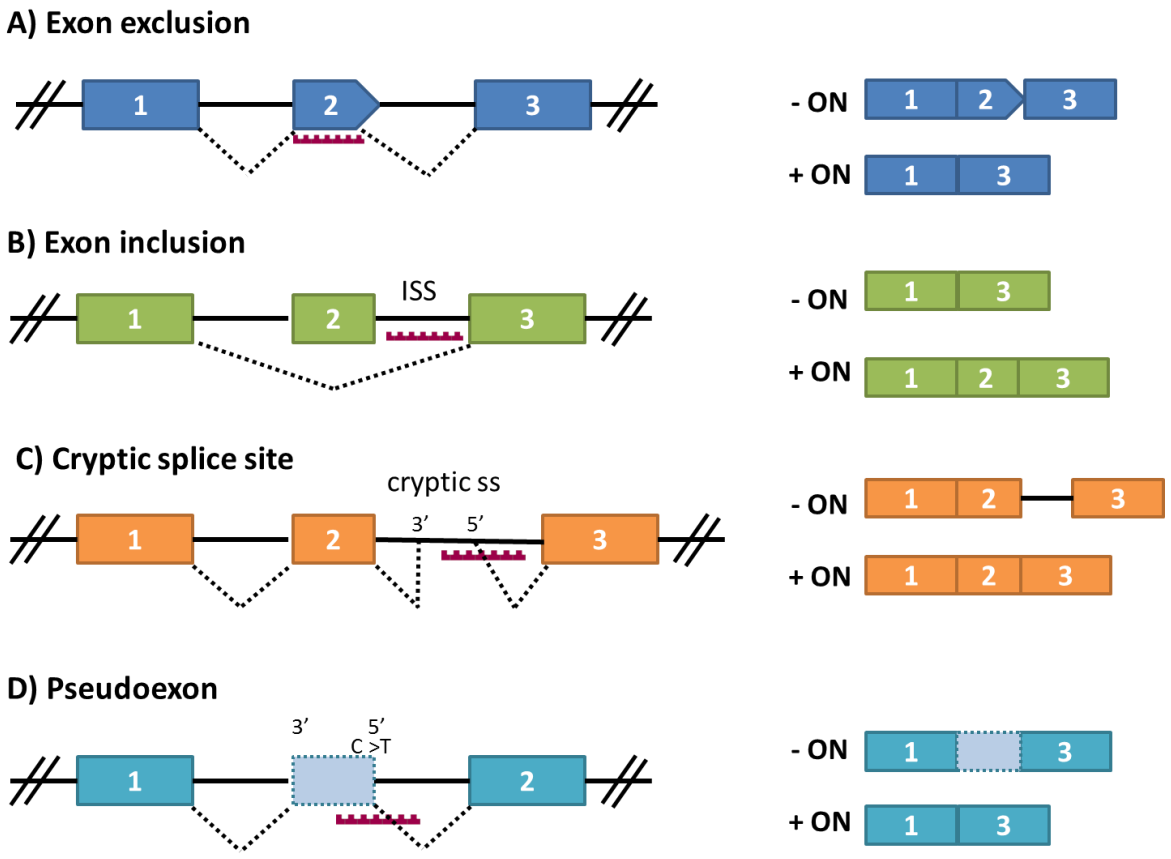

Figure 2: Splice modulation therapies. A) Large-scale deletions can result in out-of-frame mRNA. ONs can mediate the exclusion of the exon and reformation of an in-frame transcript. **B)** ONs can also be used to modulate the incorporation of exons, which are predominantly excluded from mRNA transcripts forcing the use of alternative 5'-splice site. **C)** Cryptic splice sites are weak recognition sites used only when a mutation disrupts the authentic splice site, by creating a 5´ss which activates a cryptic 3´ss. They can be found within an intron or exon, resulting in the inclusion of intron parts or partial deletions of exons from mRNA, respectively. ON targeting the mutated 5´ss, blocks recognition by the splicing machinery and generates transcripts without the aberrant intron element **D)** Pseudoexons, or false exons, are intronic sequences with strong but unused 5´ and 3´ss at either end. These sequences are infrequently found in the mature mRNA sequence. Exclusion of pseudoexons from mRNA can be attributed, in part, to silencer elements and the secondary structure of the pre-mRNA. Pathological pseudoexons arise from mutations that create a splice donor or acceptor site

either de novo or within a weak and normally unused splice site. Other mutations resulting in the incorporation of pseudoexons include the generation of novel branch point sites and creating or deleting splice regulatory elements that bind trans-splicing enhancers or silencers. (-----) stands for abnormal splicing.

SSOs or SCOs are typically 15-30 nucleotides long and have to be completely modified with chemical modifications (combined or not) that improve binding affinity and stability, including e.g. 2´-OMe PS, 2´-MOE PS, LNA PS, and PMO [69,75,141] (**§ 1.4**). Shown to work in animal models include, PNA oligomers, alternating LNA and deoxynucleotide ONs, fully modified 2′-MOE ONs, fully modified 2′-OMe ONs and PMO-based oligomers [141].

The efficiency of ONs therapeutics as splice modulators (SSOs and SCOs) has been reported for several diseases including cancer [84], metabolic [144], neurodegenerative [115], inflammatory, immunodeficiency and viral (Ebola). The therapeutic application of SSOs is most advanced in DMD but, the same compounds are also being studied as potential treatments for spinal muscular atrophy (SMA) and β-thalassaemia, as well as RA [141].

1.3 OLIGONUCLEOTIDE THERAPIES – GENOMIC APPROACHES

ONs have also been developed to target genomic DNA and thus regulate the gene expression at the transcriptional level (**Figure 1**). Like RNA therapeutics (**§ 1.2**), also the anti-gene approach is based on sequence-specific targeting using DNA- or RNA-based ONs.

Four mechanisms have been reported for gene expression regulation by ONs at the genome level. Through binding of triplex-forming ONs (TFOs) to the major groove of dsDNA (**§ 1.3.1**); invasion of DNA duplex (**§ 1.3.2**); chromatin remodelling and epigenetic gene silencing by miRNAs [78,145] (**§ 1.2.1**); or destabilisation of non-B DNA structures and R-loops (**Figure 5**, **§ 1.3.3**).

1.3.1 Triplex-forming oligonucleotides (TFOs)

The possibility for formation of DNA triplex structures was suggested by Pauling and Corey [146] and demonstrated experimentally by Fesenfeld *et al.*[147] by showing that poly(U) and poly(A) strands in a 2:1 ratio were capable of forming a stable complex in the presence of Mg^{2+}. DNA triplex structures are formed at polypurine-rich regions of dsDNA, through binding of triplex forming ONs (TFOs) to the major groove of the duplex forming Hoogsteen hydrogen bonds [148] or reverse Hoogsteen hydrogen bonds, in parallel or antiparallel orientation, respectively [149].

Parallel triplexes are formed by TFOs with TC or GT motifs, originating d(T:A-T), d(C:G-G) and d(C:G-C+) triads formed through Hoogsteen hydrogen bonds. Antiparallel triplexes are typically formed by purine (GA) or mixed (GT) TFOs forming d(T:A-A), d(C:G-G) and d(T:A-T) triplets through reverse Hoogsteen hydrogen bonds [43,150] (**Figure 3**).

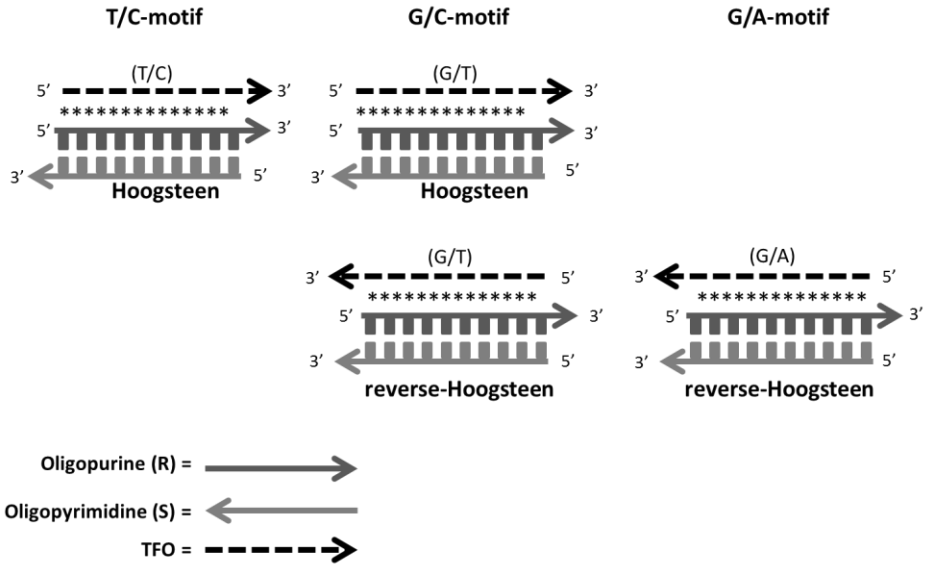

Figure 3: DNA triplex structures with parallel and antiparallel orientation.

Consequently, the success of TFOs as anti-gene therapeutics is dependent on the density and genomic localisation of specific polypurine:polypyrimidine-rich regions, designated as triplex target sequences (TTS). Bioinformatics and wide analysis studies have shown that TTS are over-represented in the human genome at regulatory regions, especially in promoter zones [151]. Structurally, TTS are significantly more curved and rigid than normal DNA, suggesting that they act as spacing fragments, helping in the correct positioning of transcription factors [152]. 97.8% of known human genes have at least one potential high-affinity TTS in the promoter and/or transcribed gene regions and 86.5% of these genes have at least one TTS that is unique for that gene [153].

Three TFO applications have been reported: interference with DNA repair (site-directed mutagenesis, site-directed recombination) *in vitro* and *in vivo* [154–157]; location of DNA-mutagenic compounds with direct site-specific DNA damage [46,158,159]; and modulation of gene transcription [160,161].

TFOs can modulate gene transcription (e.g. in c-myc [43], ets2 [162], ICAM-1 [163]), inducing down-regulation of genes by preventing protein binding to DNA [164,165]; and by inhibiting DNA replication (e.g. in simian SV40 [166] and HIV-1 [167] virus) [168].

While promising, TFO antigene strategies show low intracellular efficacy, probably due to cellular environment conditions. Like any RNA therapeutic ON, TFO needs to be taken up by cells (**§ 1.5**) and be resistant to nucleases. Moreover, they need to be able to form triplexes at physiologic pH, overcome charge repulsion between TFO and dsDNA, endure the chromatin environment and be stable enough to compete with endogenous proteins [169]. The use of PNA (**§ 1.4.3**) and LNA (**§ 1.4.2**) chemical modifications seem to overcome the nuclease resistance, the efficient binding and triplex formation challenges, at least *in vitro*.

1.3.2 Invasion of DNA duplex

Another mechanism in antigene strategies is the invasion of DNA duplex by ONs and includes three different approaches *in vitro* (**Figure 4**).

1) Invasion of the DNA duplex and binding of a linear ON to the sense-strand by Watson-Crick (WC) bonds, leaving the antisense-strand displaced;
2) Invasion of the DNA duplex and binding of the ON simultaneously to both sense- and antisense strands by WC bonds;
3) The TFO-arm of the "bis" ON binds via Hoogsteen hydrogen bonds to the sense-strand, while the WC-arm strand invades the duplex and hybridises with the same strand. The antisense strand is displaced.

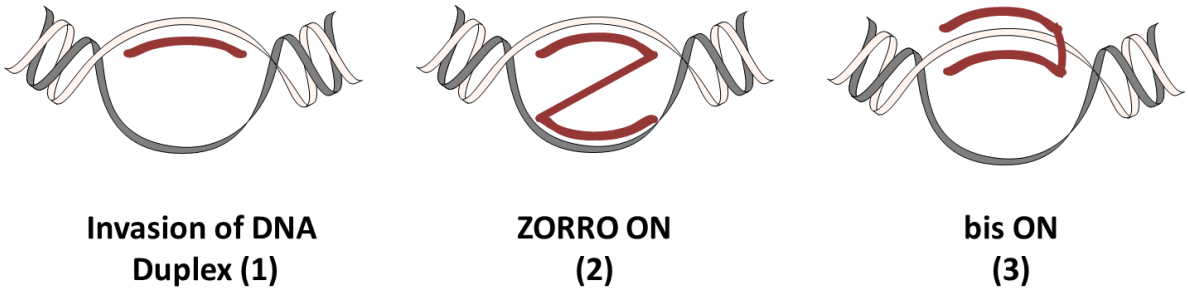

Figure 4: **Schematic representation of the three invasion approaches of DNA duplex.**

Invasion of DNA duplex (**Figure 4 (1)**) associated with transcription inhibition was reported for the first time for linear PNA oligomers targeting the tandem CAG repeats and leading to transcription inhibition of the androgen receptor and TATA-binding protein genes. The analysis of the corresponding transcripts confirmed the progression arrest of the RNA polymerase complex in both directions outside the location of PNA/DNAhybridization [170]. Down-regulation of the *c-myc* gene was also obtained when a PNA ON conjugated to a nuclear localisation signal (NLS) peptide, was targeted to a unique sequence located in the *c-myc* second exon [171]. Inhibition of the human progesterone and androgen receptors expression was also achieved and reported by Corey and co-authors, using linear PNA [172]

(**§ 1.4.3**), LNA [173] (**§ 1.4.2**) and peptide-conjugated PNA [174] oligomers targeting the promoter regions.

The second approach for DNA duplex invasion (**Figure 4 (2)**) was developed in our lab using LNA ONs (**§ 1.4.2**) targeting both sense- and antisense-strand. In this construct, a 14-mer arm binds to the sense strand, while a 16-mer arm binds the antisense strand. The two arms can hybridise (5'-end with 3'-end) through a bridge of seven base pairs, acquiring a Z-like structure (Zorro-LNA) [175]. Zorro-LNA demonstrated good strand invasion in plasmids, and the reporter gene transcription was blocked in transfected cells. The construct was further optimised to be smaller and easier to design, resulting in a 3'-5'-5'-3' single-stranded Zorro-LNA (ssZorro) by using both 3'- and 5'-phosphoramidites [176]. This new construct shows a higher rate of double-strand invasion (DSI) as compared to Zorro-LNA.

In 2011, Ling *et al.* reported the use of Zorro-LNA targeting a single CTCF binding site at the neurofibromatosis gene locus and consequent alteration of long-range DNA interactions in human fibroblast cells [177]. Recently, Bohländer *et al.*, using synthetic ON, reported that this type of invasion approach also works when two pyrrolidinyl PNA oligomers are used [178].

The third approach is based on "bis" ONs (**Figure 4 (3)**), composed by a TFO-arm and a WC-arm connected by a linker. In this concept, the TFO-arm recognises the polypurine region of the target site, while the WC-arm is responsible for the stand invasion of the DNA duplex and hybridization to the same strand. This mechanism was first reported for bisPNA oligomer in a plasmid context [179,180] and recently in our lab using bisLNA ON [181,182]. Interestingly, McNeer *et al.* (2015) described, *in vivo*, the correction of the cystic fibrosis transmembrane conductance regulator (CFTR) gene F508del mutation, using bisPNA oligomer and a donor DNA, delivered in biodegradable polymer nanoparticles [183].

1.3.3 (De)stabilisation of non-B DNA structures and R-loops

Non-B DNA structures refer to all DNA conformations, other than the orthodox right-handed Watson-Crick structure, including distorted bond angles or unpaired nucleotides [184,185]. Non-B DNA structures include triplexes, cruciforms, G-quartets (G-quadruplex, G-tetraplex or G4-DNA), and slipped structures (like hairpins), among others [184] (**Figure 5**). Their formation is dependent on the DNA sequence, the orientation of the DNA strands and length. They can be found in promoters, 5' UTRs, exons, introns and 3' UTRs [186]. Intramolecular triplex structure, so-called H-DNA, forms at mirror repeat sequences through dissociation of half of the repeat double helix and "folding back" of one of the single strand produced. This rearrangement leads to the formation of a triple helix, stabilised by Hoogsteen or reverse Hoogsteen hydrogen bonds, and a single strand region (**Figure 8**, **§ 1.7**). Cruciforms are formed in inverted repeats (i.e. palindromic sequences) of more than six nucleotides long. G-quadruplexes are non-helical structures formed by guanine tetrads interacting by Hoogsteen

bonds, stacked and stabilised by monovalent cations. Slipped DNA forms in DNA repeats with no spacer and creates a hairpin in both DNA [149,187].

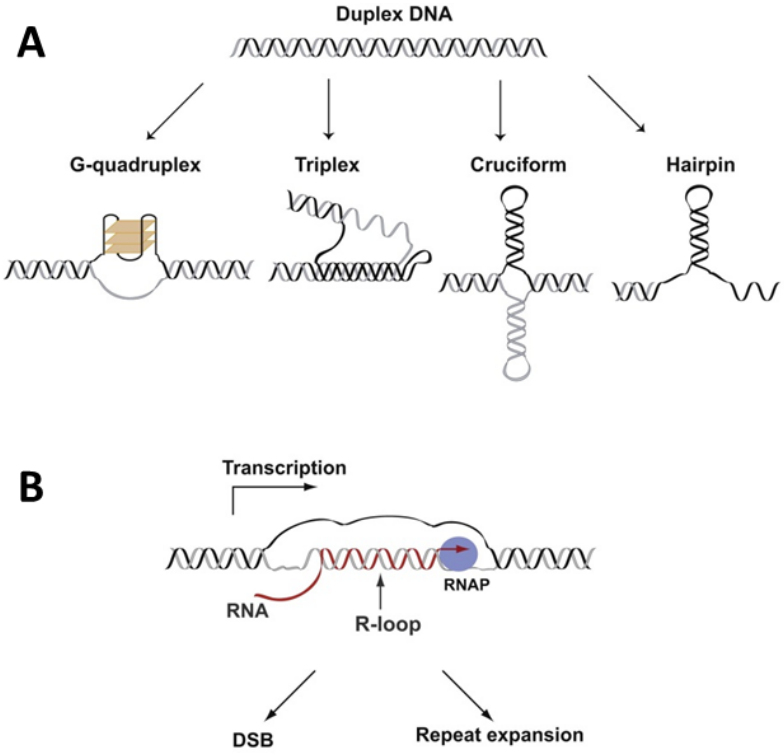

Figure 5: Non-B DNA structures and R-loops. A) The formation of secondary structures or the presence of the sequence motif per se can lead to double-strand breaks (G-quartets, triplexes, cruciforms and hairpins); induction (G-quartets and GAA/TTC tracts) or inhibition of transcription (G-quartets and triplexes); initiation (triplexes) or stalling of replication (G-quartets, triplexes, cruciforms and hairpins); and sequestration of cellular proteins (G-quartets, triplexes and r(CUG) hairpins). **B)** R-loop could be formed due to the persistent interaction of nascent pre-mRNA and the repetitive DNA template during transcription, leading to repeat expansion and chromosomal fragility. (Adapted with permission, Biochimie 95(2), 117-123, copyright 2013) [188]

Several studies *in vitro* and *in vivo*, show that non-B DNA structures can interfere with the formation of the nucleosome formation, binding and elongation of RNA polymerase II, or interaction of specific factors with their target DNA sequences, hence promoting or inhibiting transcription [187]. Additionally, they can also interfere with DNA replication and trigger genomic instability [188]. Thus, these structures contribute to the generation of polymorphisms, to genome evolution and development of a variety of diseases [184,185,188].

At least two classes of human genomic disorders are strongly related to non-B DNA structures. Approximately 20 hereditary neurological diseases can be associated with simple sequence amplifications, while ~50 hereditary diseases are caused by, for example, genomic rearrangements and gross deletions. Less well studied, but linked to polymorphisms of simple repeat sequences, are some psychiatric diseases (such as schizophrenia, drug and alcohol abuse, attention-deficit or hyperactivity disorder, and anorexia-bulimia) (reviewed in [186]).

Another nucleic acid structure found in the genome is the R-loop. It is composed of a DNA–RNA hybrid and a displaced single-stranded DNA [189] and can, putatively, be formed due to the persistent interaction of nascent pre-mRNA and the repetitive DNA template during transcription. The precise mechanism by which R-loops form *in vivo* is still unclear, although three models have been proposed [190]. R-loops are more abundant than any other non-B DNA structures in mammalian genomes. They possess crucial roles in transcription and chromatin structure, but can also contribute to genome stability. In cells, several factors and processes limit or prevent R-loop formation and accumulation. They can be removed by either ribonucleases or helicases or proteins involved in the DNA damage response pathways. Misfunction of these factors causes R-loop accumulation, which leads to replication stress, genome instability, chromatin alterations or gene silencing, outcomes that are frequently associated with cancer and some genetic diseases (reviewed in [189,190]).

Genomic therapies, through destabilisation/stabilisation of non-B DNA structures and R-loops by ONs, are only at an early stage. They are similar to the RNA therapeutics (**§ 1.2**) and antigene strategies (**§ 1.3**), being also based on simple nucleic acids complementarity rules. Although, is apparent that the success and efficiency of these approaches would rely on the use of chemically modified analogues or nucleic acid mimics, especially strand invasion ONs (PNA and LNA) (**§ 1.4.3**, **§ 1.4.2**). Moreover, recent studies on formation mechanisms and biological functions of non-B DNA structures has contributed to improved target design and the evaluation of drawbacks.

ONs able to invade double strand DNA have been explored to stabilise or disrupt non-B DNA structures. For example, several studies proposing and assessing the interactions, mechanisms and conditions between G-quadruplexes and PNA or PNA derivatives oligomers (**§ 1.4.3**) have been published (reviewed in [191]). Targeting of R-loops has also been reported by Nakamori *et al.*, showing that the reduction of CUG expanded transcripts, *in vitro* and *in vivo*, is achieved by repeat stabilisation at the genome level, due to reduction of R-loops using two 18-mer fully PS/LNA ONs [133] (**§ 1.4.2**).

ONs interaction with triplex DNA and R-loops is further described in **section 1.7**.

1.4 NUCLEIC ACID ANALOGUES

As highlighted in the previous sections, it is evident that the major driving force of the ON therapies is the constant development and improvement of nucleic acid analogues.

Since the synthesis of the first nucleic acid analogue, 2´-fluoro, several chemical modifications of the natural ONs have been studied. Included are modifications in the phosphodiester (PO) backbone, the sugar moiety or heterocyclic nucleobase [69,108,111]. The objective in designing new analogues is to improve specific properties such as nuclease resistance, the process of synthesis, affinity and selectivity, and in some cases the ability to cross biological membranes [69,108,110,192].

Nuclease degradation can be avoided by modifying either the PO backbone, heterocyclic nucleobases or sugar moiety [69,108]. Additionally, to increase target affinity and biological potency, modifications in the sugar moiety or heterocyclic nucleobases are used. The combination of different chemistries is also exploited for solving problems such as low sequence specificity, self-aggregation, and potential toxicity [109,110]. Cellular uptake of modified ONs can be further increased by conjugation to a wide variety of ligands: cholesterol derivatives, poly(L-lysine) tail, and shorter positively charged cell-penetrating peptides (CPPs), among others (reviewed in [108,192] (**§ 1.5**).

Different ON therapy approaches require specific or combination of chemical modifications (for examples *see* **§ 1.2 and 1.3**). Only the most appropriate chemical modifications to work included in this thesis will be presented. The chemical structures of these analogues are shown in **Figure 6**, and they are further discussed in the next sub-sections.

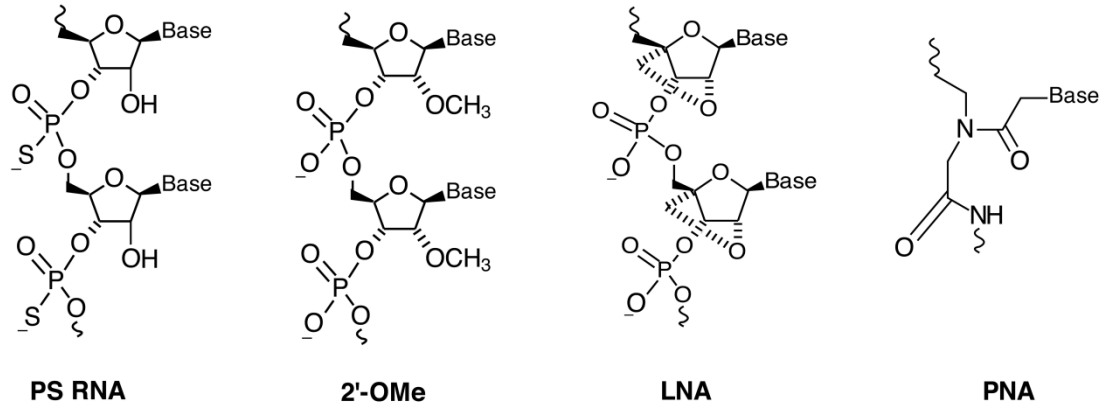

Figure. 6. ON chemical modifications. Phosphorothioate (PS) backbone [8], 2´-*O*-methyl (2´-OMe) substituent [30], Locked nucleic acid (LNA) modification [33,34], Peptide nucleic acid (PNA) [32].

Notwithstanding, additional chemical modifications are available, and constantly new ones arise.

1.4.1 2′-*O*-methyl phosphorothioate (2′-OMe/PS)

2′-*O*-methyl phosphorothioate (2′-OMe PS) ONs are an example of the so-called second-generation modifications. They combine structural modifications of the backbone linkage (PS) [8,111] with a methyl substituent in the 2′- position of the sugar moiety (2′-OMe) [30] (**Figure 6**). The PS backbone confers sufficient resistance to nuclease degradation, leading to higher bioavailability, carries a negative charge which is advantageous for cell delivery, and displays attractive pharmacokinetic properties and cellular uptake due to increased binding to plasma proteins and other receptor sites as compared to PO [111]. The 2′-OMe modification increases even further the resistance of the ON preventing endonuclease and exonuclease cleavage, increases binding affinity and modulates ON binding to proteins [108,109,193]. Fully 2′-OMe PS modified ONs prevent the RNase H1 activation and the nuclease degradation of the corresponding mRNA strand; which make them unique molecules for splicing modulation (**§ 1.2.3.1**). Furthermore, 2′-OMe PS have been extensively used as steric-blocking ONs (**§ 1.2.3**).

2′-OMe PS ONs have been successfully used in animal models targeting *apolipoprotein B-100* mRNA in Homozygous FH [113], *DM1* mRNA in DM1 [194], and *huntingtin* mRNA in Huntington's disease [195], and in human clinical trials against apolipoprotein C-III mRNA in severe hypertriglyceridemia and familial chylomicronemia [196], and against transthyretin mRNA in transthyretin-associated polyneuropathy [197].

1.4.2 Locked Nucleic Acid (LNA)

Locked nucleic acid (LNA) is an RNA analogue in which the ribose is conformationally locked by the introduction of a 2′-oxygen and 4′-carbon-methylene linkage [33,34] (**Figure 6**). The presence of this bridge promotes a conformational restriction in LNA oligonucleotides, favouring duplex formation. They also exhibit high thermal stabilities when hybridised with their RNA target molecules [108,198]. Due to the sugar conformation, LNA is resistant to 3′ exonucleases, lacks (in no-gapmer ONs) RNase H1 (**§ 1.2.2**) or RISC (**§ 1.2.1**) activity. The presence of LNA at the 3′ end of siRNA sense, or passenger, strands increases lifetime in blood, and thus potency of this ONs, but also enhances off-target effects [69,108].

Recently, LNA has been used to modify triplex forming oligonucleotides (TFOs) (**§ 1.3.1**) to increase triplex thermostability and affinity at near-physiological pH [181,182]. Like PNA (**§ 1.4.3**), LNA is also able to invade double strand DNA and binds strongly through Watson-Crick hydrogen bonds to DNA complementary sequences [181] (**§ 1.3.2**).

LNA-modified oligonucleotides have been extensively exploiting in different therapeutical areas, such as antisense mechanisms to induce RNA degradation by RNase H1 (**§ 1.2.1**), siRNA-mediated degradation (**§ 1.2.2**), altered pre-mRNA splicing (**§ 1.2.3.1**), blocking of

microRNA, and as antigene reagents to block transcription of a specific gene (**§ 1.3**) [198,199] or as decoy ON for sequestering of transcription factors [200].

1.4.3 Peptide Nucleic Acid (PNA)

Peptide nucleic acid (PNA) is a DNA mimic with an uncharged, flexible, pseudopeptide backbone composed of repeated N-(2-amino-ethyl)glycine units to which the nucleobases are linked through methylene carbonyl linkers [32,69] (**Figure 6**). PNA exhibit improved hybridization characteristics of DNA and RNA, forming very stable duplexes and triplexes with complementary ssDNA, dsDNA or RNA in a sequence-specific way [23,32,108]. This high-affinity can be explained by the lack of electrostatic repulsion due to the absence of negative charges on the PNA oligomers.

PNA is not a substrate for RNaseH and exerts its antisense effect by forming a sequence-specific duplex with mRNA, causing steric hindrance of the translational machinery, and leading to protein knockdown [69] (**§ 1.2.3**). Additionally, cell-free *in vitro* studies confirmed the potential of PNA as a sequence-specific inhibitor of transcription and translation due to the formation of the triplex complexes (**§ 1.3.1**), steric blocking of transcription factors, duplex formation at the intron-exon junction of RNAs or inhibition of viral reverse transcription. However, the application of PNA oligomers as therapeutic drugs was compromised by their poor intrinsic uptake by living cells [108,110]. Since PNAs are uncharged molecules, the majority of non-viral delivery vectors (**§ 1.5**), cannot be used. Recently nanoparticle-based strategies for efficient delivery of conventional and chemically-modified PNAs have been developed (reviewed in [201]) and proved to be effective [183].

Homopyrimidine PNA (bisPNA) oligomer, binding to dsDNA, results in a triplex invasion complex [202] (**§ 1.3.2**). The thermal stability of triplex invasion complexes is very high, but the formation is slow at physiological concentrations of salt [203]. PNAs have also been combined with intercalating moieties and nucleobases analogues (thio-pseudoisocytosine and pseudoisocytosine), to improve PNA oligomer efficiency of RNA recognition [204].

1.5 DELIVERY VECTORS

Despite the advances regarding ONs optimisation, the transfer of ON therapies from the lab to the clinics has been very slow.

The problem? ONs have poor efficacy *in vivo*. It does not mean that they “don’t work”, but simply because it is very challenging to obtain efficient delivery of such large, polar molecules to their target sites of action within tissues. Thus, the major drawback in ON therapies is still the availability of safe and efficient delivery vectors, especially *in vivo* [205].

While being the most explored and used in the gene therapy field, viral vectors are still limited by the risk of carcinogenesis, immunogenicity, broad tropism, limited DNA packaging and difficulty of vector production. On the other side, non-viral vectors are safer, can deliver higher amounts of genetic material and are easier to synthesised, but show poor efficiency in transporting the genetic material through the several cellular barriers [192,205]. Notwithstanding, recently developments in the field of material sciences (new polymers and lipids), nanotechnology (nanosized materials) and nucleic acid chemistry (an increase of potency and stability, and reduced immunogenicity), can be an option to overcome those problems.

Regarding the delivery *in vivo*, several factors have to be considered to achieve efficiency. The system must protect the therapeutic agent from degradation by endonucleases in physiological fluids and the extracellular space, avoid aggregation in capillaries or interaction with blood components, and allow selective accumulation at the tissue of interest, cellular internalisation, endosomal escape, provide transport into the nucleus (if required), and finally release of cargo [206].

ONs therapeutics can be delivered by physical and synthetic methods [207]. Physical methods include e.g. the electroporation as an option to transfect primary monocytes *in vitro*, and the injection with naked ONs which is a common choice in clinical trials. Regarding the synthetic delivery vectors, several perspectives have been explored for *in vitro* and *in vivo* delivery [82,205], like lipoplexes and lipid nanoparticles (e.g. cationic lipoplexes) [208,209] (**§ 1.5.1**); lipopolyplexes and polyplexes [210,211]; cationic polymers (linear and branched polymers, dendrimers [212] and polysaccharides); polymersomes, cell-penetrating peptides (CPPs) [213,214] (**§1.5.2**); and inorganic nanoparticles [215].

As for nucleic acid analogues section, also here only the delivery vectors related to this thesis will be described in more detail.

1.5.1 Cationic Liposomes

Lipid nanoparticles (LNs) are one of the best optimised and characterised ON therapeutics delivery systems *in vitro* and *in vivo* [216]. LNs are subdivided depending on the formulation method, which is based on the physicochemical properties of their lipids. Accordingly, they include cationic lipoplexes, neutral liposome, lipid and step-wise bulk mixing (reviewed in [216]).

Cationic lipoplexes are complexes of plasmid DNA or ONs with cationic liposomes and are the LN most used for nucleic acid delivery [211].

Cationic liposomes are composed of cationic lipids with a neutral helper lipid. Cationic lipids consist of a DNA-interacting head group with a net positive charge at physiological conditions or at lower pH; a hydrophobic lipid anchor group such as cholesterol or fatty acid

chains of various lengths and unsaturation states; and a linker group that binds the polar group to the lipidic moiety. The head group interacts with the negatively charged phosphate backbone of the ONs, thus forming a compact structure. Together with the additional neutral helper lipids, the transfection complex builds a monolayer, liposomal structure with a positive surface charge in aqueous solutions. The interaction with the cell membrane is mediated by this positive charge of the liposomes [209,217]. Following the cellular uptake by endocytosis, the lipidic moiety destabilises the endosomal membrane, resulting in a flip-flop reorganisation of the phospholipids. The endosomal phospholipids diffuse into the liposome and interact with the cationic lipids, causing the DNA dissociation and diffusion to the cytosol [215].

Lipofection is a popular transfection method and defines the process by which cationic liposomes interact with the ON spontaneously, fuse with the tissue culture cells, and facilitate the delivery of the ON into the cell. Lipofection is appropriated for cell lines and many primary neonatal cells. The technique is simple, highly reproducible, and more efficient when compared with (other) chemical methods leading to a 5- to 100-fold increased transfection rate depending on the cationic liposome. [207,208].

Although, cationic liposomes are limited to deliver only negative charged ONs (e.g. 2′-OMe PS, LNA) and plasmids. Also, they are big, show cytotoxicity at high concentrations, and demonstrate low efficacy *in vivo* delivery, owing to poor stability and rapid clearance, as well as the generation of inflammatory or anti-inflammatory responses [205,217]. Nonetheless, various liposomal formulations continue to be developed clinically.

Lipofectamine (or Lipofectamine 2000) is a 3:1 (wt/wt) formulation of the polycationic lipid DOSPA and the neutral lipid DOPE. It is commercially available and is the cationic liposome most used in cell assays for delivery of ON therapeutics and plasmids [209].

1.5.2 Cell-penetrating peptides (CPPs)

Cell-penetrating peptides (CPPs) are one of the most relevant tools for the intracellular delivery of a wide variety of cargoes such as proteins, nanoparticles, small molecules and nucleic acids (including ONs).

CPPs are usually short amphipathic or cationic (although anionic CPPs were reported) amino acid sequences with the capacity to translocate across cellular membranes [218]. They can be classified, based on their amino acid composition, as primary amphipathic, secondary amphipathic or non-amphipathic (reviewed in [214]). Despite their diversity, CPPs share common features among them. They contain natural or non-natural amino acids; are usually between 5-40 amino acids in length; carry a net- positive charge; and can transport one or more types of covalently or non-covalently conjugated bioactive cargoes over the cell membrane [219].

The major drawback of CPPs is the lack of knowledge about what exactly drives the translocation of the cargo into the cell and the mechanisms through which this occurs [218]. While non-endocytic internalisation routes have been described [220] the vast majority of the studies, to date, suggest that endocytosis is the principal mechanism for cellular uptake of CPPs. In this process, all classical endocytosis subtypes, including micropinocytosis; clathrin-mediated; and caveolin/lipid raft-dependent endocytosis have been shown to be involved [218]. CPPs present several advantages like a rapid delivery of cargoes into cells, are stable in physiological buffers and usually show low toxicity [218]. They can bypass biological membranes such as the intestinal membrane and the blood-brain barrier (BBB) [221,222] and don't are immunogenic [223].

Two clear strategies for delivery of ONs into cells are reported. The first strategy involves the synthesis of CPP-ONs covalent conjugates and yields distinct chemical entities but is often limited to short and uncharged ONs. Additionally, despite being less toxic, the transfection efficiencies are lower than those achieved for lipid-based vectors such as (Lipofectamine™ 2000). Notwithstanding, the most advanced studies in the CPP field involved the use of these conjugates for RNA splicing regulation. The second approach is based on the formation of CPP/ON non-covalent complexes or nanoparticles by electrostatic interactions. While these complexes are easy to form, their size distribution might be wide; their exact chemical composition is not well-defined and is not compatible with all CPPs. However, it enables the delivery of negatively charged ONs, such as modified and unmodified RNA and DNA, as well as RNAi effectors and plasmids. [214,219]. This strategy is less laborious and requires lower concentrations of ONs to achieve the same biological effect as with CPP-ON conjugates. One limitation to this strategy exists. Since the delivery is endocytosis-driven, much of the delivered cargo becomes entrapped in the endosomes.

In a way to enhance endosomal escape, several modifications were introduced such as the N-terminal stearic acid modification (e.g. the stearic acid-modified CPPs, stearyl-TP10) [213]. PepFect 14 (PF14) is a modified version of the stearyl-TP10, by replacement of lysines and isoleucines with ornithines and leucines. It demonstrates unusual splice-correction activity *in vitro* and can be converted into a stable solid formulation retaining the activity [224].

1.6 HYPERCHOLESTEROLEMIA – PCSK9

Hypercholesterolemia is a medical condition characterised by high levels of low-density lipoprotein cholesterol (LDL-C) in plasma [225,226]. The actual therapy is based on the use of statins, a class of cholesterol-lowering drugs [227]. While considered efficient and safe, they present some limitations at higher doses and do not sufficiently reduce LDL-C in high-risk or very high-risk subjects, requiring add-on therapies [226]. In a way to identify additional pharmaceutical targets, several genes and correspondent coded proteins involved in the cholesterol metabolism regulation were studied. One of the most promising is the

proprotein convertase subtilisin/kexin type 9 (PCSK9). *PCSK9* was discovered in 2003 [228] and identified as the third locus associated with autosomal dominant hypercholesterolemia [229].

In 2005, a study from K. Maxwell et al. demonstrate that overexpression of *PCSK9* mediated by an adenoviral vector accelerates the degradation of the low-density lipoprotein receptor (LDLR) in hepatic cells [230]. Parallel, other studies have shown the relation between gain [231–233] and loss [234] of function mutations in PCSK9 with the corresponding increase and decrease in LDL-C levels in the blood, respectively.

LDLR is the cell surface receptor that mediates the cellular internalisation of LDL-C by clathrin-mediated endocytosis for further degradation in the lysosomes, thus crucial in the cholesterol metabolism regulation. Normally, after reaching the endosome, the LDLR receptors detach from the LDL-C particles, escape from the endosome and are recycled to the membrane cell. PCSK9 is a natural post-transcriptional inhibitor of LDLR (**Figure 7**).

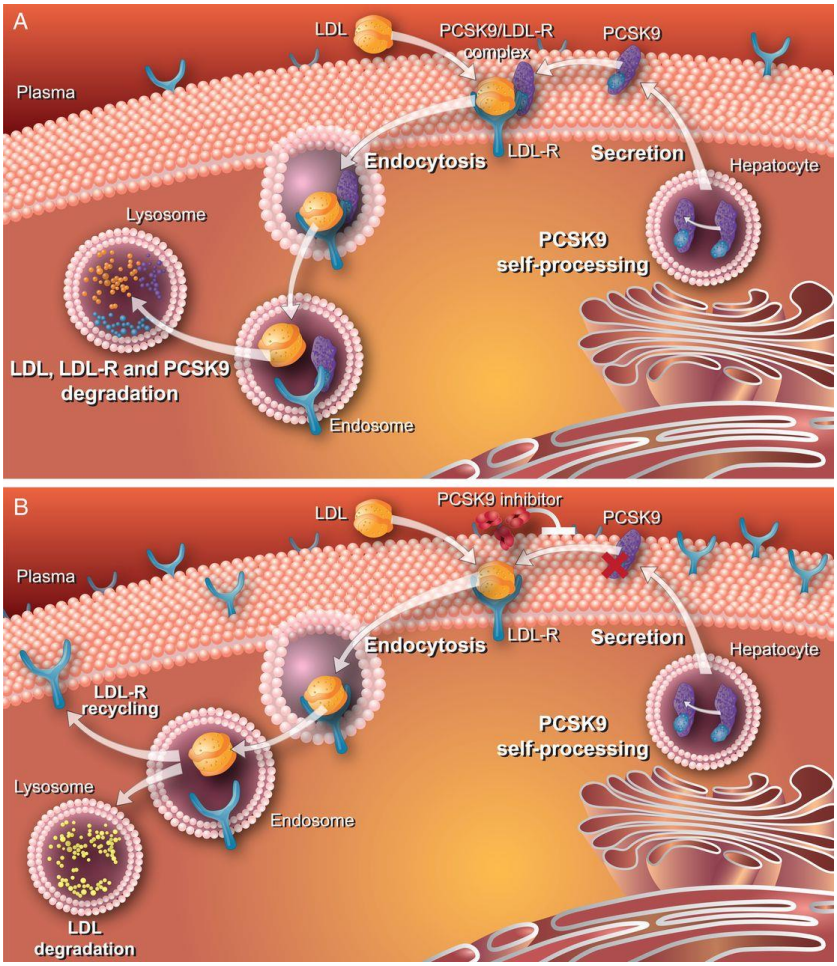

Figure 7: **PCSK9 post-transcriptional regulation of LDLR. A)** Regulation of hepatic LDL receptor expression by proprotein convertase subtilisin/kexin type 9. **B)** Mechanism of low-density lipoprotein cholesterol reduction by proprotein convertase subtilisin/kexin type 9 inhibition (Reprinted with permission from Oxford University Press: European Heart Journal 36(36), 2415-2424, copyright 2015) [250].

Briefly, PCSK9 binds to LDLR on the surface of cells and the PCSK9-LDLR complex is internalised. In the acidic environment of the endosome, the affinity of PCSK9 for the LDLR increases and LDLR is "forced" to follow the degradation pathway until the lysosomes, where it is degraded [235,236].

PCSK9 is mainly expressed in the liver, small intestine, kidney and central nervous system [237] and mediates the degradation of LDLR not only in hepatocytes, but also in fibroblasts and macrophages, but not in kidney or adrenals [238]. In 2008, Schmidt et al. described the existence of a PCSK9 splice isoform (PCSK9sv), which is expressed in multiple tissues, but contrary to full-length PCSK9 (PCSK9fl), does not show activity in controlling cellular levels of LDLR protein [239]. PCSK9sv differs from PCSK9fl by an in-frame deletion of exon 8, preventing autocatalytic cleavage of the pro-domain and secretion of the protein.

Several therapies have been developed in the last years, with variable outcomes, aiming to decrease the expression or the activity of the PCSK9 protein [240,241]. ON therapies have been explored for mRNA down-regulation by antisense ONs [242–245] or siRNA [238,246]. Antibodies-based therapies are used to inhibit the PCSK9 ability to bind to the LDLR [247,248]. Moreover, recently, a new strategy includes the disruption of the gene by introduction of loss of function mutations by a clustered regularly interspaced short palindromic repeats (CRISPR/CRISPR-associated system [249].

1.7 FRIEDREICH ATAXIA

Friedreich ataxia (FRDA) is an autosomal recessive disease, characterised by progressive ataxia, spasticity, loss of lower limb reflexes, posterior column sensory changes, scoliosis, and foot deformity. 98% of the affected individuals are homozygous for a GAA trinucleotide repeat expansion in intron 1 of the FXN gene, and concomitant low levels of FXN gene expression [251]. FXN transcription inhibition can be attributed to multiple factors including disrupted transcriptional initiation or elongation and epigenetic changes affecting chromatin remodelling and DNA methylation [252]. The reduced expression of the encoded mitochondrial frataxin protein leads to deregulated iron metabolism, mitochondrial iron accumulation and increased cellular oxidative stress.

Until now no treatments are available for FDRA. The length of the GAA repeats inversely correlates with the age of onset and directly correlates with the severity of the disease. The $(GAA)_n$ repeat is a mirror repeat DNA sequence that contains only purines on one strand and pyrimidines on the other strand. When subjected to negative supercoiling, the repeats can form intramolecular triple helix structures, generally called H-DNA [253].

It is considered that expanded $(GAA)_n$ repeats form non-B DNA structures including intramolecular triplex structures (H-DNA) (**Figure 8**), and R-loops [254]. Several other

models have been proposed for alternative structures including the formation of a higher order structure named “sticky DNA”, which consists of an intramolecular triplex(es) likely to involve a pyrimidine-motif-triplex [255]. These structures seem to affect the stability of the repeat length as well as expression of the FXN gene and may prove to be associated with promoting epigenetic chromatin changes [256–258].

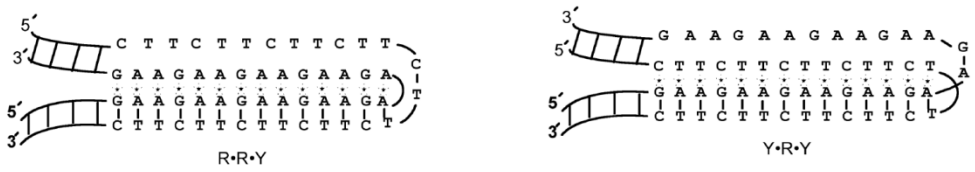

Figure 8: Purine and pyrimidine H-DNA motifs formed at (GAA)n repeats.

Therapies for FRDA are in development aiming either to revert the epigenetic changes associated with the disease, to increase Frataxin by supplementation of exogenous mature *frataxin* mRNA [259], to excise the repeats by ZFNs [260] or by (de)stabilisation of non-B DNA structures and R-loops that possible form at the repeats. Thus, histone deacetylase inhibitors were shown to increase Frataxin mRNA in FRDA mouse models and patient cell lines [255–258]; sequence-specific polyamides and low molecular weight minor groove binders enhanced Frataxin expression [253,261]; and recently Corey *et al.* reported increased expression of the *frataxin* gene by interfering with the R-loop formed at the $(GAA)_n$ repeats using LNA ONs in patient cell lines [262].

Regarding the triplex DNA, Bergquist *et al.* reported an increase in the proportion of the triplex formed at $(GAA)_{115}$ repeats in plasmids in the presence of a $ss(CTT)_n$ DNA ON, suggesting stabilisation of the structure. However, the amount of triplex was not affected in the presence of the corresponding $ss(GAA)_n$ DNA ON [263].

All the work presented here and in the section 1.3.3, is the first proof-of-concept for each of these structures. Thus it would not be surprising to expect new developments in the field of non-B DNA-structure targeting in the future time.

2 AIMS

This Ph.D. thesis aimed to develop and improve the applicability of ON therapeutics in different perspectives:

I. Use of ON therapeutics as agents to modulate the proportion of two natural splice variants to regulate the gene expression and consequently the amount of active protein (Paper I).

II. Development of new tools for screening ON chemistries and delivery vectors in a cell type dependent context (Paper II).

III. Development and characterization of a new nucleic acid analogue with cell uptake properties independent of delivery vectors (Paper III and IV).

IV. Evaluate the capacity of ONs to resolve non-B DNA structures in the genome, which are associated with gene silencing and consequently diseases (Paper V).

3 METHODOLOGY

This section describes theoretical and practical aspects regarding the most relevant methods employed in the different papers presented in the thesis. A more detailed description of the protocols and additional methods can be found in the original documents.

3.1 CELL CULTURE

Cell lines are the gold standard of all "*in vitro*" experiments for biological studies, as well as screenings and development of drugs. Characterised by their simplicity, species-specificity, convenience and possibility for automation, they are simple models of complex biological systems as a whole organism, preceding the animal trials [264]. A cell line consists of a population of cells derived from a tissue of a multicellular organism, with the ability (acquired or induced) to proliferate indefinitely in culture and share a genetic profile among them.

Different cell lines derived from human and mouse tissues were employed in this work. The human liver hepatocellular carcinoma cell lines HuH7 and HepG2 were used in the screening and activity evaluation of *PCSK9* SSOs (Paper I). For the development of the new pLuc/705 splice-switching reporter cell lines (Paper II) the HuH7, the human osteosarcoma U-2 OS, the mouse myoblast C2C12 and the mouse neuroblastoma Neuro-2a cell lines were chosen. In the study for determination of cell type efficiency characterization of already establish and new oligonucleotide chemical modifications and delivery approaches (Papers II and IV), the human cervix adenocarcinoma reporter cell line HeLa Luc/705 was used, together with the HuH7_705, U-2 OS_705, C2C12_705 and Neuro-2a_705 cell lines. For the evaluation of oligonucleotide cellular uptake by confocal microscopy (Paper III and IV), the U-2 OS cell line was the option.

All cell lines were maintained and cultivated in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) high glucose, GlutaMAX™ supplemented with 10% fetal bovine serum (FBS, Life Technologies) at 37ºC, 5% CO_2 in 95% humidity. For the new reporter cell lines, an additional supplementation of the medium with 400 or 200 µg/mL of Geneticin was made.

3.2 ANIMAL EXPERIMENTS

NMRI female mice with 20–22 g were used in the animal experiments in Paper I to access the splice-switching modulation ability of human sequence-specific *PCSK9* SSO *in vivo*. The experimental protocol was approved by the Stockholm South Ethical Committee, according to the guidelines of the Swedish National Board of Laboratory Animals at Karolinska Institutet, permit number S48-12. The experiments were performed according to the ethical permission and designed to minimise the suffering and pain of the animals.

Mice, while anaesthetized, were treated with a 2 mL hydrodynamic infusion to the liver through the tail vein, or left untreated. Hydrodynamic infusion is a method known to result in preferential delivery to the liver and subsequent high expression [265,266]of the reporter plasmid. The infusion liquid consisted of a 0.9% NaCl solution containing 5 μg of reporter plasmid pLIVE.PCSK9.Luc together with either 0.7 mg/kg SSO or 3.7 mg/kg SSO. The next day, livers were carefully harvested post-mortem and snap-frozen for subsequent analysis.

3.3 TRANSFECTION

Transfection is the process by which natural or synthetic nucleic acids (e.g. plasmids, siRNAs, and oligonucleotides) are introduced into eukaryotic cells by non-viral methods [207]. The transfection efficiency is dependent on several conditions such as transfection media, cell type and density, nucleic acid concentration and the delivery method chosen [214,216]. Consequently, depending on the objective, several protocols can be followed.

For the evaluation of the activity of *PCSK9* SSOs (Paper I), cells were seeded the day before transfection, to have approximately 80% confluency (or 50-60% confluency for the starvation experiment) the next day. Transfection of the SSO constructs and the siRNA (positive control) was performed with Lipofectamine® 2000 (Life Technologies) or Lipofectamine® RNAiMax (Life Technologies), respectively and according to the manufacturer's protocols. For the SSO constructs, a ratio of 2.5 μL of Lipofectamine® 2000 per 1 μg of SSO was used. The transfection complexes were left in the culture for 24 h, after which medium was changed, or cells were harvested.

For the establishment of the stable reporter cell lines (Paper II), the cell lines, seeded the day before and at a 80% confluency, were transfected with 1.5 μg of the pT2Kole-Neo together with 0.5 μg of the pCMV(CAT)T7-SB100 vector with Lipofectamine® 2000 (Life Technologies) for HuH7 and U-2 OS cell lines, or Lipofectamine® 3000 (Life Technologies) for C2C12 and Neuro-2a cell lines following the manufacturer's protocol. The plasmid constructs were used at a ratio of 2 μL of Lipofectamine® 2000 per 1 μg of plasmid, or a proportion of 2.2 μL of Lipofectamine® 3000 and 2 μL of P3000™ reagent per 1 μg of plasmid. Twenty-four hours post-transfection, the medium was removed, and new medium was added. Forty-eight hours post-transfection, the medium was changed to selection medium (DMEM plus 10% FBS supplemented with 800 μg/mL (HuH7, C2C12 and Neuro-2a cell lines) or 600 μg/mL (U-2 OS cell line) Geneticin antibiotic (Life Technologies)) to initiate the isolation of monoclonal cultures.

In the transfection of the reporter cell lines with SSOs by PepFect14 (Paper II), the cells were seeded the day before transfection, to reach approximately 70-80% confluency at the time of transfection. SSOs were mixed with PepFect14 at 5:1 molar ratios in nuclease free water in 10% of the final treatment volume (i.e. 50 μL). Nanocomplexes were formed for 30 minutes at room temperature. Before the addition of the complex to the cells, the medium in the wells

was replaced with fresh medium (450 µL). The complexes were left in the culture for 24 h, after which cells were harvested.

When the reporter cell lines were transfected with SSO by Lipofectamine® 2000 (Paper II), the same protocol conditions described for the Paper I (see above) were used.

3.4 pLuc/705 SPLICE-SWITCHING REPORTER

Developed by Kang et al. [267], the pLuc/705 splice switching reporter (represented by the reporter cell line HeLa pLuc/705) was fundamental in the development of the field of oligonucleotide therapeutics, and even today continues to be extensively used in the development of new nucleic acid analogues as well as delivery vectors.

It consists of a *luciferase*-encoding gene interrupted by a mutated β-globin intron 2. The existence of this mutation (T→G in the position 705) of the intron creates an aberrant 5' splice site that activates a cryptic 3' splice site, resulting in aberrant splicing of *luciferase* pre-mRNA and the translation of non-functional luciferase. When an SSO masks the aberrant site, splicing is redirected generating the correct mRNA, and consequently, the luciferase activity restored (**Figure 9**) [267].

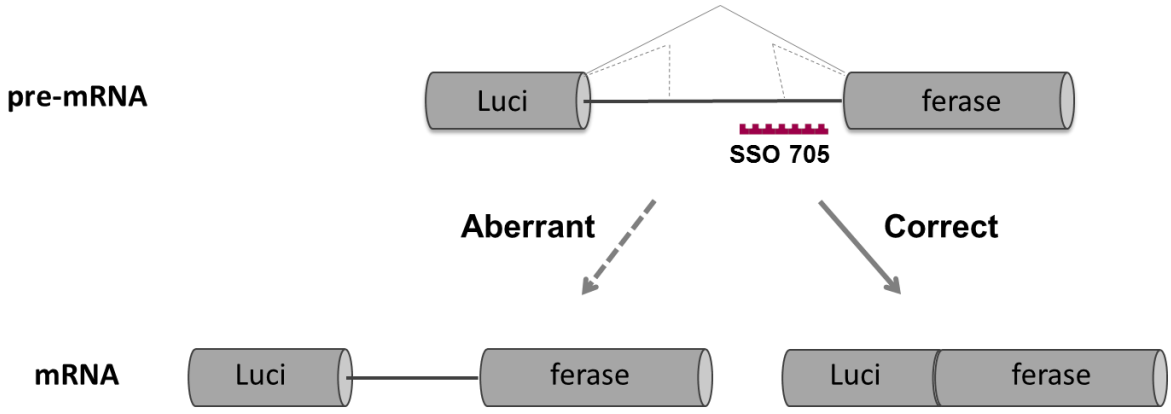

Figure 9: Schematic representation of the pLuc/705 splice-switching reporter.

This reporter was chosen for the development of the new stable reporter cell lines derived from muscle, neuron, liver and bone cell lineages (Paper II). The new reporter cell lines, named C2C12_705, Neuro-2a_705, HuH7_705 and U-2 OS_705, together with the well-known HeLa pLuc/705, were further used for the characterization of nucleic acid chemical modifications, RNA analogues and delivery vectors (Paper II and IV).

3.5 GENE EXPRESSION ANALYSIS

3.5.1 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a variation of the polymerase chain reaction (PCR) commonly used for the analysis and quantification of mRNA expression. It comprehends the conversion of mRNA in a more stable cDNA by a reverse transcriptase enzyme, followed by exponential amplification of the target gene using specific primers flanking the region of interest. RT-PCR can be performed in a one-step or a two-step reaction, and the final products can be quantified through two RT-PCR categories: endpoint (relative, competitive and comparative methods) or real-time (SYBR Green, TaqMan Probes, Molecular Beacon Probes) [268].

While both one-step (Paper I) and two-step (Paper I and II) reactions were used in the present work to amplify the different mRNA splice forms of *PCSK9* and *luciferase* genes, in all cases quantification was obtained with end-point protocols and through the relative method.

For a relative quantification the co-amplification of internal control is required, commonly an endogenous gene like the housekeeping genes *HPRT* (Paper I), *GAPDH* or *18S* (Paper II). Its expression level should be unaffected by the experiment conditions, constant across all samples and the same cellular abundance as the target mRNA. This internal control serves to normalise the samples, after which a direct comparison of relative transcript abundances can be inferred among samples.

The normal/alternative and correct/uncorrected splice products, as well as the endogenous control, were identified by their size using an agarose gel electrophoresis and band intensities were quantified using the QuantityOne Software (Bio-Rad).

3.6 PROTEIN ANALYSIS

3.6.1 Western Blotting

Western Blotting is a semi-quantitative technique in cell and molecular biology that allows the separation and identification of proteins from a mixture, based on their molecular weight. After electrophoresis, the proteins are transferred to a nitrocellulose membrane, which is further incubated with proper primary and secondary antibodies to allow visualisation [14,269].

This technique was used in Paper I to quantitatively measure the increase of the PCSK9 splice variant (PSCK9sv) and the LDLR, after cell treatment with SSOs.

The membranes were scanned using the Odyssey Classic equipment (LI-COR) and fluorescence intensity of the secondary antibodies determined by the software Image Studio 4.0. For quantification, the fluorescence intensities were plotted against the amount (μg) of loaded protein and the slope of the curves calculated by the method of least square. By this

approach, the slope of the curve, describing the regression between the fluorescence signal and the amount of protein loaded, reflects the concentration of the protein of interest.

3.6.2 Luciferase assay

Characterised by its sensitivity and linear response range, this bioluminescent assay is built on the enzymatic activity of firefly luciferase protein to oxidise D-luciferin in the presence of ATP, oxygen, and Mg^{2+}, producing oxyluciferin and light, which can be measured by a luminometer [270]. This light quantitative output can then be correlated with the amount of firefly luciferase protein produced.

However, to achieve greater confidence, data must be normalised before comparisons. Normalisation removes sample-to-sample variability caused by factors other than those being tested in the experiment, like variabilities in cell plating and transfection efficiency, pipetting inconsistencies, and toxicity [271]. Several methods can be used for normalisation, including normalisation to total protein content (can tighten the results and is the best option for stably transfected cells), total ATP content or cell number, and normalisation with a control reporter vector.

3.7 TOXICITY ASSAYS

Toxicity assays are necessary to identify potentially hazardous chemicals and to confirm, at earlier stages of development, the absence of certain toxic properties in new promising therapeutic drugs. They can involve tests to estimate the basal functions of the cell or tests specialised for specific cell functions [272]. The most common (and used) methods are based on the measurement of specific activities characteristic of viable cells. They include the use of different classes of colorimetric tetrazolium reagents, resazurin reduction and protease substrates generating a fluorescent signal, the luminogenic ATP assay, and a novel real-time assay to monitor live cells for days in culture [273].

3.7.1 CellTiter-Glo® Luminescent Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay (Paper I) is a method to determine the number of viable cells in culture based on the quantity of ATP present. ATP has been widely accepted as a valid marker of viable cells. This correlation relies on the fact that when the cell membrane integrity is lost, the cells lose the ability to synthesise ATP and the endogenous ATPases immediately degrade any ATP remaining from the cytoplasm [273].

The CellTiter-Glo® reagent is composed of a detergent (for lysing of cells), ATPase inhibitors (stabilisation of ATP released), luciferin (substrate), and the thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that catalyses the reaction where photons of light are

generated. This system has several advantages as being homogeneous ("add-mix-measure" protocol), fast (shorter as 10 minutes), more sensitive than colorimetric and fluorometric assays, less prone to artefacts, flexible and robust (luminescence signal very stable and half-life of >5 hours).

3.7.2 Cell Proliferation Reagent WST-1

The Cell Proliferation Reagent WST-1 (Paper II) is a colorimetric assay to quantify the viability and proliferation in adherent or suspension cells. The reagent is a sterile, ready-to-use solution, which contains WST-1 (tetrazolium salt) and an electron coupling reagent diluted in phosphate buffered saline. In contact with metabolically active cells, the WST-1 is cleaved by mitochondrial dehydrogenases to soluble formazan products. The formazan dye can be measured spectrophotometrically at wavelengths 420-480 nm. The absorbance measured can be directly correlated with the number of metabolically active cells [273]. This assay due to the high solubility of the dye is more convenient, more stable and less prone to artefacts and can be used for non-radioactive, spectrophotometric quantification of cell proliferation, growth, viability, and chemosensitivity in cell populations using the 96-well plate format.

3.8 CONFOCAL LASER SCANNING MICROSCOPY FOR ON CELLULAR UPTAKE

Developed around 40 years ago confocal laser scanning microscopy revolutionises the image acquisition of biological samples. The use of specific wavelengths of light (emitted from lasers), and the use of pinholes to eliminate out-of-focus light significantly increase the ability to resolve and co-localize small structures and molecules in high resolution and contrast images [274,275].

However, this is not a straightforward technique, and there are ten important aspects to consider when using a confocal microscope, preparing a specimen, and handling digital images. First, the selection of optimal microscope components to eliminate physical factors in the design of microscopes that can result in image aberrations and can limit the resolution of pictures. Second, it is crucial to understand specimen fixation and processing, antigen–antibody interactions, fluorescence theory (quantum efficiency, quenching, photobleaching). Third, the significant advantage of confocal imaging is the acquisition of high-resolution, high-contrast images obtained through the Z-axis of a sample, and the capability of software programs to reconstruct the 3-D nature of cells and tissues. Thus, during the processing, the integrity and the 3-D architecture of the specimen should be preserved as much as possible. Fourth, a good signal to noise ratio is fundamental. Fifth, quantification of fluorescence in confocal images is tricky, and they should be interpreted as semiquantitative analysis. Sixth, a thorough understanding of how the images are collected and processed by the system

hardware is critical. Seventh, always be very detailed in sample preparation, image collection, and in handling digital images. All these steps are crucial for obtaining images of high quality and with enough information for quantification. Eighth, ninth and tenth aspects focus on the acquisition, processing and storage of the original data. [274,275].

Confocal laser scanning microscopy was used to evaluate the oligonucleotide cellular uptake (Paper III and IV). Briefly, U-2 OS cells were seeded at a density of $2x10^4$ cells per well in a 175 µm glass bottom 96-well plate (Greiner Bio-One) the day before, to be 60% confluent the next day. The fluorescein labelled ONs were diluted in warm OptiMEM (LifeTechnologies) and added to cells after medium removal. After 8 h or 24 h of incubation, the cells were processed as follows: the medium was removed, cells washed once with warm Opti-MEM and stained with a 6.25 µg/mL solution of CellMask™ Deep Red membrane stain (LifeTechnologies) in Opti-MEM, 10 min, 37ºC. After staining, cells were fixed with warm 4% paraformaldehyde, pH 7.4, in OptiMEM for 5 min at 37ºC, followed by three times washing with PBS. The cells were left in PBS and imaged immediately. Confocal laser scanning microscopy was performed using an Inverted Nikon A1R+ Confocal Microscope (Nikon Corporation, Japan) with Apo 60x oil λS DIC N2 objective (numerical aperture 1.4, refractive index 1.515) and galvano scanner. Pictures were acquired with the NIS-Elements Advanced Research Software (Nikon Corporation, Japan) using a pinhole size of 39.6 µm and Ti ZDrive performed the Z-stack bottom-to-top with approximately 0.2 µm/step.

3.9 DNA STRUCTURE

3.9.1 Triplex-directed DNA double-strand cleavage by BQQ-OP and primer extension assay

A previous study on the formation of triplex structures at FRDA $(GAA)_n$ repeats show that a low-molecular weight benzoquinoquinoxaline compound (BQQ, **Figure 10**) recognises triplex structures formed at $(GAA)_n$ repeats in plasmids. BQQ is a DNA intercalating compound that specifically binds and stabilises triplex structures of both purine and pyrimidine motifs [276–279]. BQQ is cell permeable and was shown to bind and stabilise H-DNA structures formed in plasmids in growing *Escherichia coli* cells [280].

BQQ was further converted to a triplex-specific cleaving agent, Benzoquinoquinoxaline-1,10-phenanthroline (BQQ-OP, **Figure 10**), by conjugation to a 1,10-phenanthroline derivate [277].

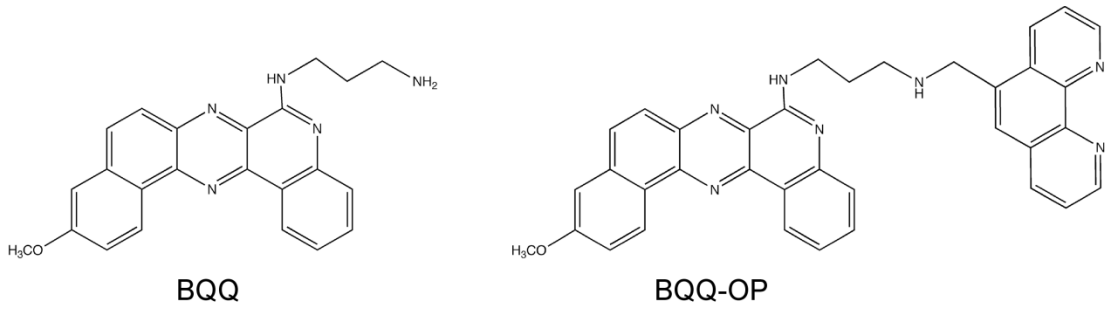

Figure 10: Chemical structure of benzoquinoquinoxaline (BQQ) and BQQ-1,10-phenanthroline (BQQ-OP).

Due to the combination of these two moieties, BQQ-OP can intercalate and cleave the double strand DNA specifically at the site of formation of a triplex (Figure 6). The reaction occurs in the presence of Cu^{2+} ions and a reducing agent, with the production of radicals *in situ*, which promoting dsDNA cleavage. BQQ-OP has been used to probe triplex formation including both H-DNA and TFO-directed triplex structures in plasmids in vitro [263,280].

The assay includes a two-step process (**Figure 11**) to analyse triplex formation in supercoiled plasmids.

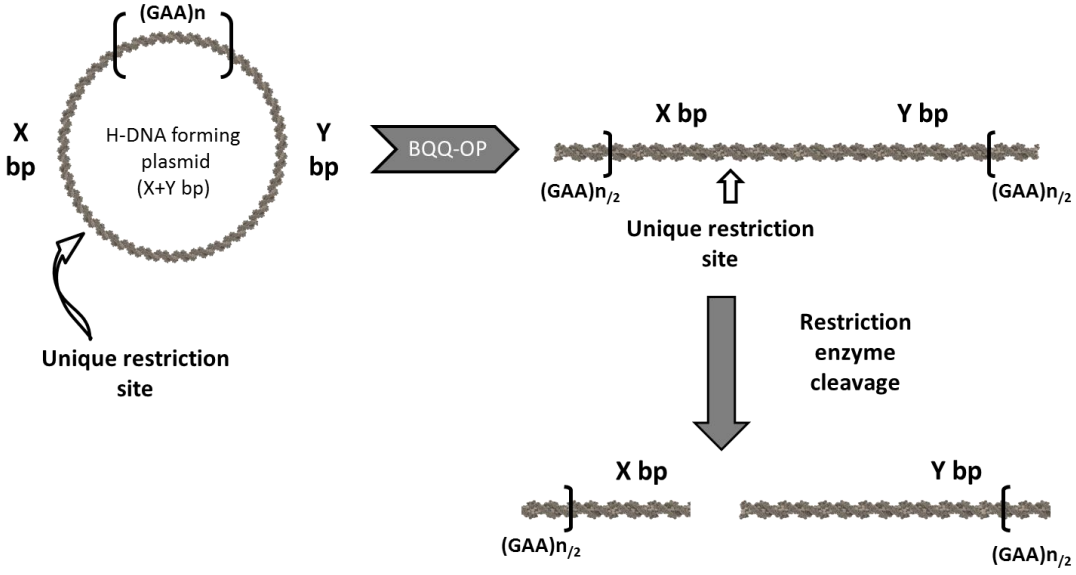

Figure 11: Schematic presentation of triplex-directed DNA double-strand cleavage by BQQ-OP. BQQ-OP binds to the H-DNA structure formed at $(GAA)_n$ repeats and causes dsDNA cleavage of the plasmid specifically at the triplex site, linearizing it. The following cleavage by a unique restriction enzyme of the plasmid yields two DNA fragments of specific sizes (X and Y) that can be resolved by electrophoresis.

First, the triplex site is cleaved by BQQ-OP linearizing the plasmid. The following cleavage by a unique restriction enzyme produces two DNA fragments of specific sizes, considering that the BQQ-OP cleavage occurs on average in the middle of the triplex. Agarose gel electrophoresis is used to resolve these two fragments, and the extent of the triplex-directed cleavage is calculated based on the intensity of the DNA bands, together with the remaining linear fragment. In the absence of triplex formation, only a linearized DNA fragment can be detected and quantified.

BQQ-OP has used to evaluate the capacity of PNA and LNA oligonucleotides to disrupt higher order DNA structures in Friedreich's ataxia $(GAA)_n$ repeats (Paper V).

A primer extension reaction is used to map the 5′ ends of DNA (or RNA) fragments. It is performed by annealing a specific oligonucleotide primer, usually radiolabelled at 5' end, to a position downstream of the 5′ end to be mapped. It is followed by DNA polymerase extension, where the DNA template is copied, and produces a fragment that ends at the 5' end of the template molecule. Analysis of primer extension of the extended DNA products is done on denaturing polyacrylamide gels and autoradiography.

Primer extension reaction was used to analyse the products of triplex-directed DNA cleavage by BQQ-OP (Paper V). The objective was to detect the nucleotides that are involved in a triplex structure. To achieve a detailed analysis of the triplex-directed cleavage of plasmids by BQQ-OP, each of the $(GAA)_n$ or $(CTT)_n$ containing strands were used as template in separate reactions. In this case, the radiolabeled primers bind to the flanking regions of the triplex forming sequence. Normally, the polymerase reaction would stop at the cleavage site on the DNA template and the fragments analysed. However, structures that may form within the DNA sequences will influence the extension reaction and may cause pausing of the polymerase. Consequently, the DNA template is linearized by a restriction enzyme to prevent structural formation and reduce pausing. Still, a background pausing is often present, which reinforces the importance of strong negative controls.

3.9.2 Atomic Force Microscopy (AFM)

Electronic microscopy (EM) has been the only analytical tool for analysis of the structure of biomolecules that are too complex for X-ray and NMR and too small for optical microscopy. Besides presenting high power and versatility, it lacks the ability to analyse structures under conditions closely resembling their physiologic environment [281]. Atomic force microscopy (AFM) emerges as a way to overcome this problem, since it can operate in air or liquid, being the only microscope that can achieve nanometer resolution on biological samples under physiological conditions. These instruments use a sharply pointed sensor, or "tip", mounted on the end of a flexible cantilever. As the sample is scanned beneath the tip, small forces of interaction with the sample cause the cantilever to deflect, thereby revealing the sample topography [281,282].

The AFM imaging acquisition from contact mode to Tapping Mode™ and to Peak Force Tapping™ reduced the damage of the specimen drastically, allowing the imaging of soft polymers, lipid bilayer, proteins, DNA, and whole cells. Furthermore, PeakForce® tapping mode, where the applied force can be controlled and decreased below the nano-Newton range, allows the analysis in liquid, an advantage compared to traditional Tapping Mode™ [283]. The possibility to perform scans in liquid is extremely advantageous for imaging unfixed biological samples near to physiological conditions [283]. However, measurements in liquid require crucial aspects in sample preparation, especially for DNA specimens.

It could be assumed that physiological condition buffers, containing Mg^{2+} and Na^{+}, both required for DNA structures stabilisation, would be enough for visualisation of DNA [283,284]. While Mg^{2+} binds very weakly to mica surfaces, the presence of Na^{+} promotes the release of DNA molecules from the surface. This salt effect can be overruled by the addition of Ni^{2+} ions to the buffer. The optimal Ni^{2+} concentration should permit an "ideal" attachment of DNA to the mica surface, avoiding Ni^{2+} precipitation and poor DNA binding, which normally occurs at higher and lower $NiCl_2$ concentrations, respectively [285,286]. Additionally, studies by Billingsley et al. (2010) demonstrate that the hydration of the sample during AFM measurements is crucial to determine the morphology of supercoiled plasmids. They show that supercoiled samples prepared with Ni^{2+} with high hydration are more condensed, with a large number of crossovers, local conformation changes, and more turns effects that seem to be topologically driven. They also postulate that under these conditions, the configuration is closer to the 3D biological shape [287].

PeakForce® Tapping measurements in liquid (Paper V) were performed on an atomic force microscope (Dimension Fast Scan, Nanoscope V, Bruker®, Santa Barbara, CA) located in the AlbaNova Nanofabrication Facility (Stockholm, Sweden). In these type of measurements, the scanner vibrates at a low frequency (1-3 kHz), resulting in a tip-sample interaction with every oscillation. The maximum force applied on the tip was decided and kept constant with a feedback loop, which adjusts the overall extension of the piezo during scanning and allows non-destructive topography imaging [285,288,289]. In this study, the AFM images were obtained using silicon nitride cantilevers with silicon tips (ScanAsyst-Fluid+, Bruker®, Santa Barbara, CA) with a nominal tip radius of 2 nm and spring constants ranged between 0.4 and 0.7 Nm^{-1}. The images were obtained with scan rates between 1 and 1.5 Hz, the maximum force of 500 pN, scanner oscillation amplitudes between 60-40 nm, scanner resonance frequency of 2 kHz, the image resolution of 512 x 512 pixels and scan sizes around 5 x 5 and 1.3 x 1.3 μm2. In all images, the vertical limit was reduced to 1 μm to enhance the resolution. Plasmid areas from all scans obtained were calculated by Image J software and GraphPad Prism 6 software performed statistical analysis.

4 RESULTS AND DISCUSSION

4.1 Paper I

RNA therapeutics inactivate PCSK9 by inducing a unique intracellular retention form.

In this paper, we aimed to develop a new approach to inactivate PCSK9, through conversion of the normal splice form to a natural, less abundant and inactive, splice variant (*PCSK9sv*) by splice-switching oligonucleotides (SSOs).

The rationale here emerged from the publication of Schmidt *et al.* [239] describing *PCSK9sv* (splice variant lacking exon 8) (**§ 1.6**) and by the fact that SSOs were previously never used for regulation of gene expression by changing the alternative splicing pattern of available biological isoforms, without creating aberrant transcription forms.

Thus, several SSOs sequences targeting different regions in the exon 8 of the human *PCSK9* gene were designed based on the data retrieved from three predictive ESE and ISE bioinformatics programs. We choose to synthesise the SSOs as fully 2´-OMe PS ONs since this is a chemical combination commercially available, transfection protocols are well characterised, and several studies show that they are efficient in splice modulation approaches and accumulate preferentially in liver and kidney.

The initial SSO screening and determination of effective SSO concentrations were initially performed in HuH7 cell lines, and the best candidates were further tested in HepG2 cell lines to evaluate if the effect is cell line specific. The expression of *PCSK9* full length (*PCSK9fl*) and *PCSK9* splice variant (*PCSK9sv*) were quantified by RT-PCR and normalised to *HPRT* mRNA. The primers were located near the extremities either on 3´-end of exon 7 or 5´-end of exon 9 to allow size discrimination between the two isoform products. We found a significant reduction of the wild-type form in both cells lines for all treatments as compared to non-treated cells ($P<0.05$ and $P\leq0.01$). Regarding the formation of the *PCSK9sv*, both cells lines show statistically significant difference between hP872 treated and non-treated groups. Together these results indicate that splice modulation was not cell line specific and that the best performing SSO was hP872 at a concentration of 100 nmol/L.

To evaluate potential toxic effects, the viability of the cells upon SSO treatment during 24 h was then measured, with no variation in viability observed for both cell lines. This experiment was also important to demonstrate that changing the proportion between the two isoforms does not affect cell metabolism.

Next, we aimed to evaluate the treatment in a more metabolic/biological context.The *PCSK9* expression is known to be regulated by the levels of cholesterol in the medium, and Medina *et al.* showed that the addition of cholesterol after a period of sterol starvation increases the full-length isoform of *PCSK9*, while it decreases the splice variant [290]. Consequently, the best way to challenge our therapeutic approach would be by confirming that the splice modulation

could remain after a period of cholesterol depletion/addition. For this purpose, Huh7 cells were treated with the SSO hP872 at 50 and 100 nmol/L for 24 h, after which, a period of cholesterol depletion, was induced for 24 h, followed by the addition of 20 nmol of cholesterol. The levels of total, full length and spliced *PCSK9* mRNA were measured after this treatment (57 h post transfection). A statistically significant difference ($P<0.0001$) is found among all treatments, and a clear reduction of the levels of the total and full-length mRNA can be observed when the cells are treated with hP872 at 100 nmol/L ($P=0.006$ and $P<0.0001$). This was also confirmed at the protein level. Concomitant with an increase in the PCSK9sv splice form, a decrease in the pro-protein form is observed when the cells are treated with the SSOs. Additionally, the data shows that, after treatment, the splice variant did not decrease as quickly as the full-length form, and its amount is significantly higher when compared to non-treated cells. This finding proves that our approach can persist against specific splice regulators and confirm the efficiency of the strategy.

To examine the biological relevance of our approach, and if an increase in *PCSK9sv* mediated by SSOs would enhance LDLR protein levels, we analysed the levels of LDLR protein in the samples of the cholesterol depletion/addition experiment. As expected, an increase in LDLR levels is observed, especially when cells are treated with SSO hP872. This final data confirm, without a doubt our hypothesis.

Once we had the proof of concept, another hypothesis arose. Is this approach also efficient *in vivo*? For this experiment, some aspects had to be taken into account. First, preliminary studies had shown that mice specific SSOs against the same sequence did not show any significant effect on the formation of the splice form. Altogether, our data suggests that this mechanism is human-specific. Second, we did not have access to relevant mice models to test our hypothesis.

To assess the human sequence-specific SSO *in vivo* a liver-specific luciferase reporter vector containing the genomic sequence of the human *PCSK9* including exon 7 to exon 9 was created, pLIVE.PCSK9.Luc. Additionally, the NMRI mouse strain was used since is considered to be the experimental animal that best mimics a human population and thus being used in many fields of general biology, in pharmacology and toxicology studies.

The mice were co-injected with the reporter plasmid and the SSO hP872 or CTR SSO by hydrodynamic infusion. The expression of the vector was verified by *luciferase* mRNA. The results show complete conversion to the splice form when SSO hP872 is present, while for CTR SSO both the full mRNA (*PCSK9_E7-9*) inserted and the splice form (*PCSK9_E7-9Δ8*) are detected. The effect was also concentration dependent. We also analysed the expression of endogenous *pcsk9,* and no significant changes are found, proving the specificity of our SSO for the human sequence.

Altogether, our data showed that *PCSK9* activity could be modulated by splice-switching through a human specific RNA therapeutic approach and the adjustment of the natural active to non-active isoform represents a physiological way of regulating the cholesterol

metabolism, by controlling the amount of LDL receptor available and the rate of LDL-cholesterol clearance.

4.2 Paper II

Four novel splice-switch reporter cell lines: distinct impact of oligonucleotide chemistry and delivery vector on biological activity.

In this paper, we aimed to develop a small "tissue" library of new stable pLuc/705 splice-switching reporter cell lines that will allow determination, if any, of the cell type dependence in the biological activity of ON chemistries and delivery vectors.

Despite the new advances in the ON field, a clear knowledge regarding how certain chemistries behave alone, or in combination with various delivery vectors, is still limited [291]. Their characterization is frequently restricted to a single reporter cell line and, when different cell lines are studied, the experimental conditions differ. Aware of this, we decided to create four new monoclonal stable reporter cell lines derived from muscle, neuron, liver and bone cell lineages carrying the pLuc/705 splice-switching reporter (**§ 3.1** and **3.4**). These cell lines, together with the already existent HeLa Luc/705, will constitute a tissue library for evaluation of new SSO chemistries and delivery vectors that allow a cell-type dependent fair comparison.

Accordingly, several monoclonal for HuH7_705, U-2 OS_705, C2C12_705 and Neuro-2a_705 cell lines were established (**§ 3.1**), expanded, selected based on the lower levels of endogenous splice correction and higher SSO-induced splice correction and characterised. Our data shows that in contrast to the previous HeLa Luc/705 cells, a slightly higher endogenous level of correctly spliced mRNA is observed in the different cell lines, but these values are similar through several passages of the cultures, demonstrating the robustness of these reporters. Equally, the total amount of luciferase mRNA is also lower, presumably as a consequence of the transposase-based approach employed here, which limits the number of insertions into the genome. The morphology of the new reporter cell lines is similar to the parental cell lines and their culture doubling times are approximately 19, 34, 28 and 21hours for C2C12_705, U-2 OS_705, HuH7_705 and Neuro-2a_705, respectively.

In order to evaluate if the influence of the ON chemistry and/or the delivery vector on the biological activity is cell type-dependent, all the new reporter cell lines together with the already existent HeLa Luc/705 reporter were either transfected with SSOs (2′-OMe PS, 2′-OMe/LNA PS and 2′-OMe-ZEN PS) (**§ 1.4.1**, **§ 1.4.2**) together with Lipofectamine® 2000 (**§ 1.5.1**), treated with PepFect14 nanoparticles (**§ 1.5.2**), or assayed for 'naked' ON uptake in Ca^{2+} enriched medium. Treatments were performed with two (and three in the naked approach) different concentrations, and both mRNA as well as luciferase production were analysed.

To exclude toxicity effects derived from the treatments, cytotoxicity for all the formulations used was evaluated, and no adverse effects were observed in any of the cell lines. Additionally, SSO formulation efficiency could be dependent on the size and number of the particles formed, and different chemistries can formulate differently. To determine if the effects would be chemistry/vector-dependent, particle size determination by nanoparticle tracking analysis was performed for all the formulations. No significant differences due to SSO chemistry were found between the size or number of particles for either delivery vector.

Excluded effects in toxicology and formulation, the readout should be mainly dependent on SSO chemistry. Previous reports in HeLa Luc/705 and confirmed in our Lipofectamine experiments, would dictate that with this delivery vector, the 705_ZEN SSO should be more potent than the 705_LNA SSO, followed by the 705_2´-OMe SSO. However, our observations clearly showed that depending on the cell line, different ON chemistries in a lipoplex context or PepFect14 nanoparticles show various activities.

Comparison of lipid-assisted with peptide-mediated delivery revealed that the 705_LNA SSO did not display any cell type variation in its activity (better in both cases in U-2 OS_705 > HeLa Luc/705 > Neuro-2a_705 > C2C12_705 > HuH7_705). In contrast, a cell type-specific pattern is found with the 705_2´-OMe SSO. Both delivery approaches show higher activity in U-2 OS_705; but then, for Lipofectamine® 2000, HeLa Luc/705 > Neuro-2a_705 cell lines, while for PepFect14, the second best is in the C2C12_705 cell line. Surprisingly, whereas the 705_ZEN SSO is the best SSO in all cell lines when delivered using the cationic lipid reagent, its activity is very low (U-2 OS_705 and C2C12_705) or negligible using PepFect14 nanoparticles. The reason for this variability remains unknown and requires further study.

Cell line dependence of different chemistries is also found in ‘naked’ uptake. The 705_LNA SSO is more efficient in cell lines considered as poor in ‘naked’ uptake, like HeLa Luc/705, Neuro-2a_705 and C2C12_705, while the 705_2´-OMe and 705_ZEN SSOs perform better in cells like HuH7_705 and U-2 OS_705. Our data is in agreement with the *in vivo* findings for low activity of 705_ZEN in muscle cells upon naked systemic delivery of the SSO. Most interesting is the low activity of SSOs like 705_LNA and 705_ZEN in the HuH7_705 hepatocyte cell line, leading us to postulate that the higher activities normally observed for these SSOs *in vivo* in liver could be a consequence of the high vascularization of this organ or the profound fenestration rather than a sign of cell specificity or other fundamental differences between primary hepatocytes and this laboratory adapted cell line.

In conclusion, we successfully establish a new tissue library of reporter cell lines. Through them, we demonstrate that the biological effect of ONs is dependent on the particular chemical modification as well as on the delivery vector, both activities being cell line dependent. Thus we believe that these new cell lines have high potential as models for cell type-specific screening in the ON field. Additionally, the biological effect can be easily evaluated by RT-PCR or by a Luciferase assay, allowing for high-throughput analysis.

4.3 Paper III

Nuclease resistant oligonucleotides with cell penetrating properties.

In this paper, we report the synthesis and characterization of ONs modified with a new nucleic acid chemical analogue 2′-*O*-(N-(aminoethyl)carbamoyl)methyl (2′-*O*-AECM) adenosine.

The groundwork started in the lab of Prof Roger Strömberg with the synthesis of the first 2′-*O*-AECM adenosine analogue and the formation of 2′-*O*-AECM adenosine dinucleotides [292]. It was shown that the 2′-*O*-AECM moiety, to an even greater extent than the carbamoylmethyl (CM) modification [35], protected the dinucleotide against enzyme-catalysed degradation by snake venom phosphodiesterase I, and made it completely resistant to degradation by spleen phosphodiesterase II.

The question if 2′-*O*-AECM ONs could be synthesised, and what properties they would have, was raised based on the high nuclease resistance shown by the 2′-*O*-AECM dinucleotides, together with a study showing that ONs containing the N-((N,N-dimethylaminoethyl) carbamoyl)-methyl modification could form RNA duplexes with higher melting temperatures.

This work started with the synthesis of 2′-*O*-AECM adenosine containing ONs with several lengths (17, 16 and 13-mer), number and position of the modifications. This ONs were further evaluated for stability, type of interaction and conformation in the context of RNA or DNA duplexes. The results show a general increase in the thermal melting of the complexes RNA (+0.5 to +2.3 degrees per modification), while in complexes with DNA there was little difference (-0.3 to +1.0 degrees per modification) compared to a DNA-DNA duplex. Additionally, the slightly thermal melting increase observed for 2′-O-AECM:DNA is mainly due to electrostatic interactions, while for 2′-*O*-AECM:RNA the stabilisation is not only an electrostatic effect, but additional factors could stabilise de duplex. Structurally, the incorporation of the 2′-*O*-AECM modification forces the duplex (with DNA or RNA) to adopt an A-conformation, which is consistent with the higher melting temperature. Moreover, the stability to nucleases was also evaluated by incubation with 90% human serum. HPLC data showed that after 24h, the 13-mer fully modified 2′-*O*-AECM ON was still intact.

Cellular uptake of ONs without the addition of transfection agents is rare. As discussed before, CPP-ONs conjugates (**§ 1.5.2**), due to the positive charge of the peptide moiety, can efficiently be delivered to cells. The 2′-*O*-AECM modification also possesses a positive charge, resembling a cell penetrating oligonucleotide (CPO), raising the question if they will be freely taken up by cells.

In that respect, a 10-mer 2′-*O*-AECM adenosine ON fluorescein labelled was synthesised and tested for naked cellular uptake in U-2 OS cells at 8μM. Confocal microscopy analyses showed an efficient cellular uptake after 8 h incubation. However the spotted appearance could indicate that most of the material was still entrapped in the endosomes.

Our results show the successful synthesis of 2′-*O*-AECM adenosine containing ONs, which demonstrate a positive effect on the thermal melting of duplexes with RNA, resistance to degradation in human serum and cellular uptake without any additives, such as cationic lipids or cell penetrating peptides.

4.4 Paper IV

Fully and partially AECM-modified oligonucleotides. Synthesis and initial studies on cellular uptake and splice-switching activity in different reporter cell lines.

In this manuscript, we aimed to evaluate the potential of 2′-*O*-AECM modifications in fully and partially modified ONs as splice-switching therapeutic agents.

The interesting findings from Paper III lead us to further explore the 2′-*O*-AECM modification in a biological context. The development of the new reporter cell lines from Paper II, together with the existent reporter cell line HeLa Luc/705, dictated the biological approach and the ONs sequences to be used here.

The 705 SSO has a mixed sequence, including all four nucleotides. Since our previous report on 2′-*O*-AECM modified ONs only involved the adenosine monomer, the synthesis of 2′-*O*-AECM guanosine, uridine and cytidine building blocks were developed. The synthesis of the 18-mer fully 2′-*O*-AECM modified ON (**ON1**) and its derivatives also required synthesis improvements. The **ON1** was analysed regarding the target mRNA, showing an increase in melting temperature of 0.4ºC per modification, which is similar to the value of a 2′-OMe PS.

Further studies, as compared to Paper III, to verify the influence of the 2′-*O*-AECM modification on cellular uptake using confocal microscopy were done. Thus the synthesis of a series of fluorescein-labelled fully modified ONs of various length, ONs incorporating a mix of 2′-O-AECM and 2′-*O*-Me groups, and ONs with a combination of 2′-*O*-AECM/ 2′-*O*-Me PS or 2′-*O*-AECM PS/ 2′-*O*-Me PS chemistry was performed. Similarly as in Paper III, also the U-2 OS cell lines were used, but the ON concentration was reduced to 4 µM and two-time points tested, 8 and 24h. The changes in the protocol were made to verify if the same behaviour would be observed at lower concentrations (8 µM is not a desirable concentration for naked uptake).

Confocal microscopy analysis revealed that the 2′-O-AECM modified ONs clearly have the ability to be taken up by cells, in the absence of transfection agents, and that this internalisation is ON size- and modification-degree dependent. The data from the combined ONs (AECM, PS and 2′-OMe), showed that after 8h the positive control (fully 2′-OMe PS ON) showed high cellular internalisation, some nuclear localisation and a more diffuse pattern, while the 2′-*O*-AECM containing ONs showed lower uptake and appeared to have higher endosomal localisation. No significant changes were observed between the 2′-*O*-AECM containing ONs. Additionally, at 24 h, an increase in the uptake was observed for all

ONs. Data show that the fully 2′-OMe PS ON has a nuclear localisation exclusively. On the other hand, AECM modified ONs showed a relation between the increase in uptake and nuclear localisation, with an increasing amount of PS backbone linkages. This data set suggests that the incorporation of PS backbones improves the uptake and cellular distribution of 2′-O-AECM modified ONs. Notwithstanding, the comparison between the two-time points seems to indicate that a different uptake mechanism for the AECM modification may also be involved. Also and as previously reported for PS ONs, 2′-O-AECM modified ON internalisation and distribution within cells can also be time, temperature, concentration and cell line dependent.

To assess the splice-switching activity, several ONs containing AECM modifications were synthesised and tested: fully AECM; combined 2′-*O*-AECM/2′-*O*-Me; combined 2′-*O*-AECM/2′-*O*-Me oligonucleotides with complete PS backbone; and combined 2′-*O*-AECM/2′-*O*-Me with partial PS backbone. The splice-switching activity was evaluated in the new reporter cell lines (C2C12_705, HuH7_705, Neuro-2a_705 and U-2 OS_705) (Paper II) in parallel with the commonly used HeLa Luc/705 [267] and with a calcium-supplemented protocol [293] at 1 or 4 μM after 72 h. The splice-switching activity was measured by luciferase readout.

The data show no significant increase in the luciferase levels for all ONs in the "difficult to transfect" C2C12_705 cell line. So, additional measures are needed for efficient splice-switching in this cell line, as also showed in the Paper II. Regarding the other cell lines, a statistically significant concentration-dependent increase of the luciferase levels was found for AECM containing ONs. For the hepatocyte HuH7_705 and the cervix HeLa Luc/705 cell lines, a higher spice-switching activity was found with the 2′-OMe PS ON, while significant splice-switching was found with AECM containing ONs, especially for fully AECM modified ON. Based on the results, for these cell lines, neither replacement of AECM nucleosides with 2′-*O*-Me nucleosides or introduction of PS adds any benefit regarding the activity. The same is true with the Neuro-2a_705 cell line, where especially the fully AECM modified ON and the partially PS/partially 2′-*O*-Me ON displayed a similar activity as the 2′-OMe PS ON.

It is interesting to see that with U-2 OS_705 cells, although all ONs show significant activity there is a beneficial effect of adding PS to the backbone of AECM oligonucleotides. This is consistent with the increase in cellular uptake in U-2 OS cells observed by confocal microscopy. However, the similar splice-switching activity of the 2′-OMe PS ON and the AECM oligonucleotide with the partial 2′-*O*-Me and full PS modification ON, at higher concentration, is not quite consistent with the confocal data. Here, the 2′-OMe PS ON gives a somewhat higher uptake. This can be possibly explained with the different time points, 24 versus 72 h, used for the confocal experiments and the splice-switching assays. Confocal experiments seem to indicate that the uptake kinetics could be somewhat slower for the AECM ONs than for the 2′-OMe PS ON, but there are also substantial differences in the experimental conditions. However, both experiments support the conclusion that

incorporation of PS in the AECM oligonucleotides is beneficial in U-2OS cell lines. However, for the other cell lines tested, this effect was not observed.

The ONs *Tm* when hybridising with the target mRNA were also measured. No indication of the T*m* values of the ONs can explain the differential effect on the splice-switching activity. This indicates that the different activities may be cell type dependent or due to intracellular availability/uptake of ONs, rather than to their affinity for the target.

In conclusion, we showed that AECM modified ONs could be synthesized and combined with previously established chemical modifications. We confirmed that 2′-O-AECM modified ONs clearly have the ability to be taken up by cells, in the absence of transfection agents. We also found that 2′-O-AECM modified ONs can act as splice-switching ONs in several cell lines (HuH7_705, HeLa Luc/705, Neuro-2a_705, U-2 OS_705 or C2C12_705).

4.5 Paper V

Disruption of higher order DNA structures in Friedreich's ataxia $(GAA)_n$ repeats by PNA or LNA targeting.

In this work, we aimed to disrupt using sequence specific PNA oligomers or LNA ONs, the non-B DNA structure(s) formed at the $(GAA)_n$ repeats in the *FXN* gene and associated with Friedreich's ataxia.

This work emerged as a continuation of the report from Bergquist et al., in which it was demonstrated that CTT DNA TFOs bind dsDNA at GAA repeat sequences forming a pyrimidine motif triplex. Conversely, purine motif triplex formation was not detected at this site when using the corresponding GAA TFO [263]. Additionally, it was also demonstrated that binding of a single strand CTT ON to the H-DNA forming plasmid enhanced triplex-formation significantly, whereas an analogous GAA ON had no such effect.

Knowing the high capacity of PNAs to form triplex structures, we decide to test if PNA would behave differently when targeted to FRDA expanded GAA repeats. PNA oligomers consisting of CTT or GAA repeat sequences were used. Triplex formation by PNA oligomers was examined using a triplex-specific dsDNA cleavage reaction mediated by BQQ-OP (**§ 3.9.1**) in linearized plasmids to avoid intrinsic H-DNA formation.

The data show that binding of CTT-PNA (in presence or absence of Mg^{2+}) followed by BQQ-OP dsDNA cleavage resulted in the formation of two DNA fragments having the expected sizes indicating the formation of a triplex. In contrast, binding of GAA-PNA shows only a single DNA fragment exhibiting slightly slower gel mobility than the linearized plasmid, which can indicate stable PNA binding to the dsDNA plasmid, maybe due to the formation of a duplex invasion complex. Based on these observations and that the triplex formed by CTT-PNA binding resulted in a clear BQQ-OP cleavage, we conclude that a purine motif triplex is not formed in the presence of GAA-PNA.

Similarly, binding of CTT and PNA oligomers to FRDA repeats in supercoiled pMP179(115 repeats) was performed. Binding of CTT-PNA to the $(GAA)_{115}$ repeat leads to statistically significant increase of the amount of triplex, Interestingly, when a GAA-PNA oligomer is used, there is no detectable triplex-containing structure, including H-DNA. Only one major DNA fragment was observed upon BQQ-OP treatment, corresponding in size and gel mobility to the linearized plasmid. It is worth noting that a slower mobility band is detected in the sample containing the $(GAA)_{115}$ repeat plasmid and GAA-PNA indicating a stable interaction, which we believe can be attributed to a sequence-specific GAA-PNA binding to the repeats region. Our data shows that binding of a GAA-PNA oligomer to FRDA repeats has the unique ability to completely abolish all triplex structures, which are detected by BQQ-OP, including H-DNA, under these conditions.

To clarify the interactions observed previously for the PNA oligomers, structural analysis was performed using either chemical modification of ssDNA regions by chloroacetaldehyde (CAA) or BQQ-OP mediated cleavage of DNA triplex structures followed by primer extension assays. Two different supercoiled plasmids carrying short or medium $(GAA)_n$ repeats (n=9 or 75, respectively) and including FRDA flanking sequences were used.

Characterization of the structure at the shorter GAA repeat showed two different H-DNA pyrimidine motif triplex structures with the 5´3´3´ H-DNA isomer as the predominant isomer. Binding of CTT PNA is compatible with the formation of a triplex invasion complex of two CTT-PNA oligomers binding to the R-strand at the $(GAA)_9$ repeat. On the other side, binding of GAA PNA showed a clear formation of a single strand region throughout the R-strand and is therefore ascribed to a GAA-PNA duplex invasion of the repeat region.

Regarding the characterization of the medium GAA repeat, we could only detect the of formation of a 5´3´3´- pyrimidine H-DNA. In this case the chloroacetaldehyde modification of the longer repeat failed to reveal bands that could indicate the presence of non-base paired nucleotides in either of the R- or Y-strand, suggesting the presence of more complex higher order DNA structures and indicating a difference between the pathological and normal repeat lengths. Binding of CTT PNA suggests three different modes of interaction: CTT binds to the single-strand region in the H-DNA, formation of a triplex invasion complex of two CTT-PNA oligomers (consisted with the 9 repeats data) or triplex formation. The GAA PNA data showed, as for the 9 repeats, a simple strand invasion.

Based on these results it is clear that PNA oligomers can interact with H-DNA formed at the $(GAA)_n$ repeats and while CTT PNA stabilises this structure GAA PNA seems to disrupt it.

To examine whether another type of modified oligomer, with comparable dsDNA invasion properties as PNAs, could mediate results similar to those previously observed, the BQQ-OP cleavage assay was performed in the presence of CTT- or GAA-LNA-PS oligomers. The results show that in the presence of GAA LNA ONs the amount of H-DNA is almost not detectable, in contrast to the treatment with a 12-mer CTT LNA oligonucleotides. This results support the hypothesis that modified GAA ONs can be used to destabilise triplex structures formed at $(GAA)_n$ repeats.

DNA topology is affected by the formation of higher order DNA structures not the least at expanded triplet repeats. Atomic force microscopy (AFM) has been used to examine

cruciforms, H-DNA and higher order structures at CAG and GAA repeats, respectively. In a similar way, we reasoned that oligomer binding to structure forming GAA expansions should be reflected by changes in DNA topology (and thereby morphology of circular DNA) and could be detectable by AFM. To examine this hypothesis, we monitored the effects of LNA binding to pMP179(115 repeats) using CTT-, GAA- or a scrambled-LNA (**§ 3.9.2**).

The results showed clear changing in the plasmid morphology when GAA oligonucleotides were present, acquiring a more relaxed morphology when compared to non-treated plasmid or controls. To our knowledge, this was the first time that a plasmid of this size and characteristics was analysed by AFM in liquid.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

The development of new technologies and approaches for ON therapies are the main focus of the present thesis.

This work contributes to the field of ON therapeutics by an emphasis on the major challenge that is the delivery. First, the reporter cell lines developed will enable the chance to characterise chemistries and vectors in cell types for which reporters were not previously available. Determining which chemistry is suitable for different cell types will allow the development of new therapeutical approaches with the potential of organ targeting.

The new nucleic acid analogue with cell penetrating properties has the potential to facilitate the delivery of ONs in the naked form, i.e. without the needed for delivery vectors. However, further studies are required until the full potential of this chemistry is unravelled.

We also present two different approaches for gene regulation, namely at the RNA and at the DNA level. While promising, the full therapeutic potential for hypercholesterolemia using RNA splice-modulation of the two isoforms of PCSK9 is not known. More studies are needed in relevant animal models. However, in theory, this approach represents a novel concept, which could either stand-alone or serve as a complement to other cholesterol-reducing treatments.

The first steps for targeting non-B DNA structures formed at the GAA repeats with ONs have been taken. To reveal their full potential studies need to be carried out in a more relevant biological context. Under the conditions used, i. e. *in vitro*, outside cells, PNA seems to be more efficient than LNA; however, in a biological context, LNA may prove to be better, because of its more favourable uptake into cells. Recent developments in technologies for PNA delivery may selectively promote the uptake. Further studies are needed to develop this concept into a more physiological approach.

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